REMARKS

This application has been amended in a manner that is believed to place it in condition for allowance at the time of the next Official Action.

Claims 89-91 and 98-100 are pending in the present application. Claims 89-91 have been amended to address the formal matter raised by the Examiner in the outstanding Official Action. Claims 98-100 have been added. Support for new claims 98-100 may be found in original claims 89-91.

In the outstanding Official Action, claims 89-91 were rejected under 35 USC §112, first paragraph, for allegedly failing to comply with the enablement requirement. This rejection is respectfully traversed.

At this time, the Examiner is respectfully reminded that it is a well founded principle that any assertion by the Patent Office that the enabling disclosure is not commensurate in scope with the protection sought must be supported by evidence or reasoning substantiating the doubt so expressed.

As a matter of law, the expressed teaching of the patent specification cannot be controverted by mere speculation and unsupported assertions on the part of the Patent Office. As stated by the Court of Customs and Patent Appeals in the case of *In re Dinh-Nguyen and Stanhagen,* 181 USPQ 46 (CCPA 1974):

> Any assertion by the Patent Office that the enabling disclosure is not commensurate in scope with the protection

> sought must be supported by evidence or reasoning substantiating the doubt so expressed. 181 USPQ at 47.

Such a standard must be applied with great care when the Examiner's conjecture is contrary to the teachings of the specification.

Upon reviewing the Office Action, applicants believe that the Official Action fails to meet its burden in showing that the claimed invention is not enabled by the present disclosure. Indeed, the Official Action does not provide any evidence to support its position that the disclosure does not enable a method of suppressing the differentiation of blood precursor cells and hematopoietic stem cells in vitro using Serrate-1 peptides comprising SEQ ID NOs: 5, 6 or 7.

Indeed, while the Official Action alleges that colony formation suppression (decrease of numbers of colonies) does not correlate to differentiation suppression, applicants believe that one of ordinary skill in the art would conclude that differentiation suppression can be extrapolated or inferred from the results set forth in Examples 10, 11 and 12 found in the present specification. Contrary to the assertions of the Official Action, one of ordinary skill in the art would find that the assay methods used in the present invention can be used to study the differentiation suppression of hematopoietic cells.

The colony assay in Example 10 correlates to the assays found in the article by GORDON, *Human Haemopoietic Stem Cell*

Assays, "Direct Clonogenic Assay, Colony forming Units for Granulocytes, Erythroid Cells, Monocytes and Megakaryocytes (CFU-GEMM) (pg. 191-192), and the article by MOORE, *Clinical Implication of Positive and Negative Hematopoietic Stem Cell Regulators*, "BFU-e, CFU-GM, CFU-GEMM" (pg. 2). In particular, the Examiner's attention is respectfully directed to the article by GORDON at pg. 191, wherein it is stated that

> Clonogenic assays are the most directly quantitative means of measuring human haemopoietic progenitor cells in vitro. Haemopoietic colonies are essentially clones of cells produced by a single progenitor cell. The colonies can be analysed morphologically and by replating the cells they contain into different clonogenic systems to obtain information about the self-renewal and differentiation potential of the colony-forming progenitor.

Thus, as demonstrated by these publications, the assays of the present examples detecting progenitor cells in undifferentiated blood cells can be used to determine differentiation suppression. Indeed, the results of example 10 indicate suppression of colony formation (small number of colonies) as compared with the results obtained when Serrate-1 is not added.

Moreover, the colony formation after long term liquid culture (LTLC) in Example 11 of the present invention corresponds to an assay utilized by GORDON, *Human Haemopoietic Stem Cell Assays*, "Secondary Colony Formation, Long-term Bone Marrow Culture (LTBMC) and Long-term Culture-initiating Cells (LTCIC) (page 193)". This shows the colony formation of cells after a

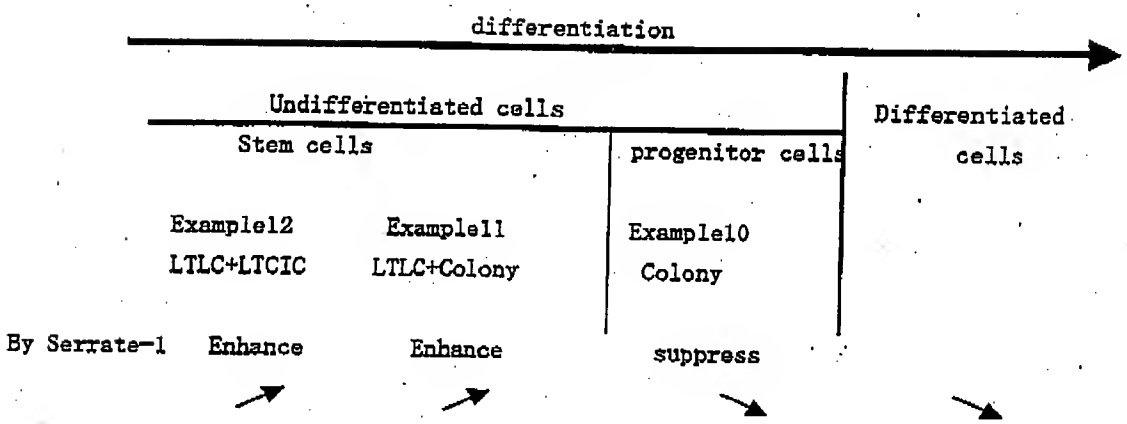
long-term culture (secondary colony formation). As a result, numbers of colony forming cells after the culture were found to be in larger amounts than with conditions that add MIP-1α but do not include Serrate-1.

In Example 12, long-term culture initiating cells (LTCIC) after LTLC were examined. Example 12 shows that the numbers of LTCIC (frequencies) were larger than those without adding Serrate-1.

Relationships of these results are compared and are expressed in the following table with reference to Figure 1 of MOORE, *Clinical Implication of Positive and Negative Hematopoietic Stem Cell Regulators*, "BFU-e, CFU-GM, CFU-GEMM" (pg. 2).

As shown in Figure 2 of METCALF, *Hematopoietic Regulators: Redundancy or Subtlety?*, with regard to the synergistic effect of cytokines, a colony can be interpreted as binary, i.e., it is indicated that proliferation is indicative of an increase in the size of the colony, and the increase in the number of colonies is a result of additional precursor cells.

Table 1 Analysis of Result on Examples 10-12



Thus, the differentiation proceeds from the left to right direction. The differentiation generally proceeds during colony formation. It can be observed from Examples 11 and 12 that an increased number of stem cells can be observed by the addition of Serrate-1. It can also be observed from Example 10 that colony formation is decreased by an addition of Serrate-1 in progenitor cells. The increase and decrease (fluctuation) in numbers of cells reflects the suppression of differentiation from stem cells to progenitor cells (transition from the left to the right direction). In other words, the number of stem cells in the precursor step is increased due to suppression of the differentiation to progenitor cells.

Since cells in the colonies formed from the progenitor cells consist of the differentiated granulocyte, macrophage and erythroblast, the suppression of colony formation is a result of

the differentiation suppression of the progenitor cells to the differentiated cells.

Thus, as the Office Action fails to present any evidence to the contrary and the present disclosure utilizes art accepted assays to determine differentiation suppression, applicants believe that the claimed invention is enabled by the present disclosure.

Claims 89-91 were rejected under 35 USC §112, second paragraph, as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which applicants regard as the invention. Applicants believe the present amendment obviates this rejection.

In imposing the rejection, the Official Action alleged that the term "undifferentiated blood cells" was indefinite. While applicants believe that the phrase may be broad, applicants believe that the phrase is definite to one of ordinary skill in the art. Nevertheless, in the interest of advancing prosecution, the phrase "undifferentiated blood cells" has been deleted in favor of the phrase --blood precursor cells and hematopoietic stem cells--. Support for this change may be found in the present specification at page 10, lines 1-10.

In view of the present amendment and the foregoing remarks, applicants believe that the present application is in condition for allowance. As a result, applicants request the allowance and passage to issue of the present application.

The Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 25-0120 for any additional fees required under 37 C.F.R. § 1.16 or under 37 C.F.R. § 1.17.

Respectfully submitted,

YOUNG & THOMPSON

Philip Dubois, Reg. No. 50,696
745 South 23rd Street
Arlington, VA 22202
Telephone (703) 521-2297
Telefax (703) 685-0573
(703) 979-4709

PD/lk

APPENDIX:

The Appendix includes the following items:

- Gordon, Human Haemopoietic Stem Cell Assays
- Moore, Clinical Implication of Positive and Negative Hematopoietic Stem Cell Regulators
- Metcalf, Hematopoietic Regulators: Redundancy or Subtlety?

BLOOD
REVIEWS
State of the Art

Human Haemopoietic Stem Cell Assays

M.Y. Gordon

SUMMARY. **Assay systems for human haemopoietic stem cells are necessary for understanding the regulation of haemopoietic cell production. The stem cells are ultimately responsible for the production of mature blood cells but constitute only a tiny minority of the total haemopoietic cell population. The remainder is a heterogeneous mixture of cells at various stages of differentiation and maturation along the myeloid, erythroid and megakaryocytic lineages. Although clonogenic assays for the lineage-committed progeny of human stem cells are well established and have produced a wealth of information about the regulation of the later stages of haemopoiesis, there is no universally accepted assay for self-renewing pluripotent haemopoietic stem cells in human haemopoietic tissue. Nevertheless, several types of in vitro and in vivo assay systems have been devised for very early haemopoietic cells in man. These can be broadly divided into three classes: (1) direct clonogenic assays; (2) assays where non-clonogenic cells are detected by their ability to produce more mature clonogenic progeny and (3) transplantation of human haemopoietic cells into immunocompromised (SCID) mice. The different assay systems are designed to answer important questions about the control of haemopoietic stem cell proliferation and differentiation and to provide qualitative and quantitative information about stem cell numbers and identity.**

Kinetics of Haemopoietic Stem Cells

The process of haemopoiesis is often visualised as a series of compartments of increasing maturity, frequently displayed as a unidirectional linear branching hierarchy (Fig. 1). In normal steady-state haemopoiesis, the size of the stem cell population is maintained at a constant level by the balance between stem cell production by cell division and stem cell loss by differentiation. This means that, in order to obtain a net increase in stem cell number, the probability of self-renewal must be increased to more than 0.5. This is clearly possible in vivo, as demonstrated by the regeneration of haemopoiesis following myelosuppression, haematological reconstitution following bone marrow transplantation and the expansion of the stem cell population during fetal development. However, the conditions needed to obtain an increase in stem cell numbers in vitro have not been adequately defined.

The probability of stem cell self-renewal at the single cell level is thought to be a random process and, consequently, it is not possible to predict whether an individual stem cell will self-renew or differentiate.[1] In contrast, at the stem cell population level, the overall picture is one of controlled self-renewal and differentiation that appears to be tightly regulated so that demands for the production of differentiated cells and for the expansion of the stem cell population are met when necessary. Once cells have become committed to a particular lineage of differentiation, a process that may be stochastic (i.e. the probability of entering a particular lineage is random),[2] rigidly predetermined[3] or responsive to growth factors or microenvironmental cues,[4,5] they progress through stages that can be defined by their

Dr M.Y. Gordon, Leukaemia Research Fund Centre, Institute of Cancer Research, 237 Fulham Road, London SW3 6JB, UK.

Blood Reviews (1993) 7, 190–197

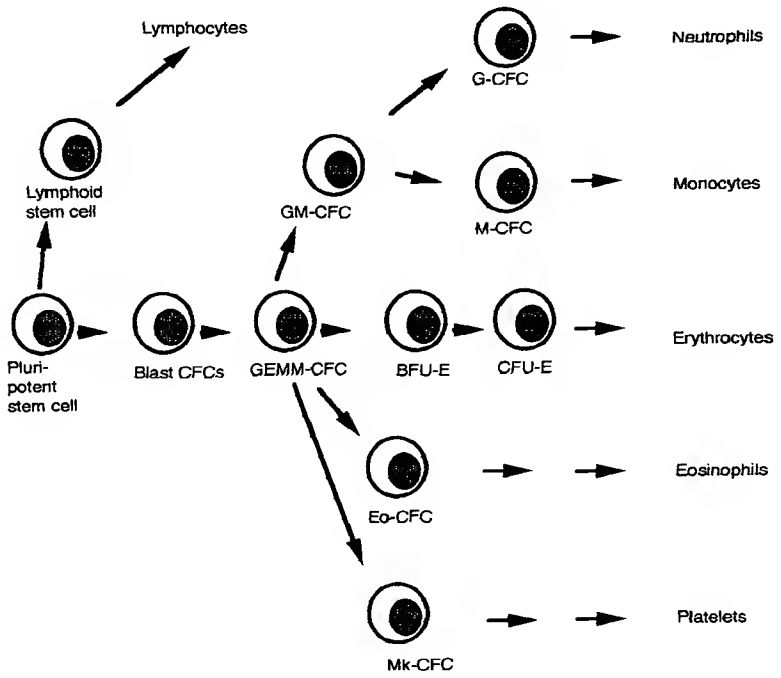


Fig. 1 Proposed hierarchy of haemopoietic stem and progenitor cells.

ability to form multicellular colonies of differentiated cells in semi-solid clonogenic assays. Finally, they lose their proliferative capacity and mature to become functional blood cells.

Stem Cell Properties

Confirmation that an assay system detects a haemopoietic stem cell population rests on several properties of the progenitor cell. Normally, the majority of the stem cells in vivo are in G_0 phase of the cell cycle and this can be demonstrated by treating cells with S phase-specific or cycle-dependent agents (^{3}HTdR, 5-fluorouracil, 4-hydroperoxycyclosphamide, cytosine arabinoside) prior to assay. Stem cells are capable of reproducing themselves (self-renewal), at least in vivo, and attempts to demonstrate this involve collecting the progeny of the candidate stem cell and replating them under the conditions used for the primary culture. They are also capable of producing cells belonging to all lineages of haemopoietic cell differentiation and this is demonstrated by plating stem cell progeny in clonogenic assays for granulocyte-macrophage colony-forming cells (CFU-GM), erythroid burst-forming units (BFU-E) and megakaryocyte colony-forming cells (CFU-Mk). In addition, candidate stem cells possess a range of phenotypic properties that can be exploited in stem cell purification and enrichment procedures. These include expression of CD34 and Thy-1,[6,7] absence of lineage-related markers[6] and transferrin receptors (CD71),[8] the high molecular weight form of the human leukocyte antigen CD45RA,[9] low expression of HLA-DR[10] and low uptake of rhodamine^{123}.[11] Physical features such as differing size and light scatter properties also distinguish cells at different stages of haemopoietic cell development.

Primitive haemopoietic progenitor cells have been selected and cultured under a variety of conditions. The aims of the experiments are to identify the characteristics of human haemopoietic stem cells and to define their potential for proliferation and lineage differentiation. Strategies for cell selection are positive or negative and a combination of both approaches is commonly used to achieve enrichment and purification. Positive selection depends on the isolation of a population of cells conforming to chosen criteria such as the expression of cell surface antigens, possession of physical characteristics, different adherence properties or pharmacological responses to drugs. Negative selection procedures enrich the cells of interest by removing irrelevant cell types. Interpretation of the results obtained is heavily dependent on the efficiency of the separation procedure used.

Assay Systems for Candidate Human Haemopoietic Stem Cells

Direct Clonogenic Assays

Clonogenic assays are the most directly quantitative means of measuring human haemopoietic progenitor cells in vitro. Haemopoietic colonies are essentially clones of cells produced by a single progenitor cell. The colonies can be analysed morphologically and by replating the cells they contain into different clonogenic systems to obtain information about the self-renewal and differentiation potential of the colony-forming progenitor.

Colony-forming Units for Granulocytes, Erythroid Cells, Monocytes and Megakaryocytes (CFU-GEMM). The CFU-GEMM are progenitors that can form colonies, in semi-solid culture medium, comprising mixtures of granulocytes, erythroblasts, megakaryocytes and macrophages (see ref 12 for review). They are not in active cell cycle in vivo and are capable of a degree of self-renewal in vitro but express HLA-DR. Thus, whilst sharing some features with pluripotent haemopoietic stem cells they may be more mature than the most primitive haemopoietic stem cells. A recent study[13] has revealed the existence in cord blood plasma of a factor(s) that could increase the replating ability (i.e. self-renewal) of CFU-GEMM in vitro which indicates, first, that the self-renewal potential of CFU-GEMM might have been underestimated and, second, that self-renewal can be modified by exposure to soluble factors.

Blast Colony-forming Cell Assay. A clonogenic assay for blast colony-forming cells has been described by Ogawa.[14] These progenitors are primitive cells and the formation of colonies by them is delayed relative to the formation of granulocyte-macrophage colonies and erythroid bursts. However, their appearance can be hastened by adding growth factors (interleukin (IL)-6 + IL-3; IL-1 + IL-3; G-CSF; IL-11; stem cell factor and leukaemia inhibitory factor (LIF)) to the culture system. In vivo, almost all of the blast colony-forming cells are out of cycle in the marrow and the action of the growth factors in vitro is thought to accelerate entry into cell cycle.

The colonies produced by the blast colony-forming cells contain new blast colony-forming cells which can be detected by replating cells from the colonies under conditions that are identical to the primary culture. This finding indicates that the blast colony-forming cells reproduce themselves during colony growth. They also contain lineage-restricted colony-forming cells that can be detected in replating experiments using the appropriate conditions for the secondary culture.

High Proliferative Potential Colony-forming Cells (HPP-CFC). The HPP-CFC were originally described in cultures of murine bone marrow and were characterised by their ability to generate very large macroscopic colonies of macrophages and their relative resistance to treatment with 5-fluorouracil in vivo. Further studies on murine HPP-CFC-derived colonies demonstrated that they contained progenitors of granulocytes, erythrocytes and megakaryocytes and that cells from the colonies could be used to repopulate haemopoiesis in vivo in mice.[15] McNiece and colleagues[16] have described a progenitor population, in human marrow, with similar properties to the murine HPP-CFC. Their frequency in normal marrow is about 2 per 10^5 mononuclear cells.

Srour et al[17] asked whether human HPP-CFC are capable of expansion in vitro. They initiated stroma-free long-term cultures with CD34-positive, HLA-DR-negative rhodamine 123^{dull} cells and maintained them with c-kit ligand (stem cell factor) and a synthetic IL-3/GM-CSF fusion protein. There was a 5.5-fold increase in the numbers of HPP-CFC over a period of 6 weeks and replates of these colonies revealed that some of them contained further HPP-CFC. A plausible explanation of these results is that the initiating cells were capable of self-renewal.

CFU-A. These cells form large mixed lineage colonies in agar culture. There are up to 250 of these progenitors per 10^5 mononuclear cells in human marrow. They have a low replating capacity (3%) for macroscopic colony formation but a high replating efficiency (50%) for producing small colonies of neutrophils and macrophages. Like the CFU-GEMM and HPP-CFC, the majority of the CFU-A appear to be non-cycling cells in vivo. They appear to occupy a position in the haemopoietic cell hierarchy that is intermediate between the CFU-GEMM and the CFU-GM.[18]

Colony Formation on Stromal Feeder Layers. Cohen et al[19] reported that colonies of adherent haemopoietic cells (cobblestone areas) are produced when murine marrow is added to preformed stromal layers. Following up this observation, Gordon et al[20] added human bone marrow mononuclear cells to preformed stromal layers and identified a population of blast colony-forming cells (BI-CFC) that adhered to the stroma and formed colonies of undifferentiated blast cells. Replating of the colonies showed limited self-renewal and the presence in the colonies of CFU-GEMM, CFU-GM and BFU-E.[21] This places the progenitor in a position that is ancestral to the CFU-GEMM but further investigation of the physical, phenotypic and cytochemical properties of the BI-CFC and their progeny led to the conclusion that they are less primitive than the pluripotent self-renewing haemopoietic stem cells.[22]

More recently, Tucker, Bol and Kannourakis[23] have identified subpopulations of stroma-adherent colony-forming cells discriminated by the time of appearance of colonies on the stromal layer. They found that colonies scored after 5 days incubation, as originally described,[20] were heterogeneous with respect to their differentiation potential. As previously reported[21] further incubation of the cultures led to a fall in the number of colonies due to colony disintegration. However, separate, probably more primitive progenitors formed colonies on stroma that could be scored after 21 days in culture, placing these cells in a position ancestral to the day 5 BI-CFC.[23]

There are, therefore, a variety of clonogenic assays for early human haemopoietic progenitor cells that possibly measure closely related or overlapping populations of progenitors. This is shown schematically in Figure 2.

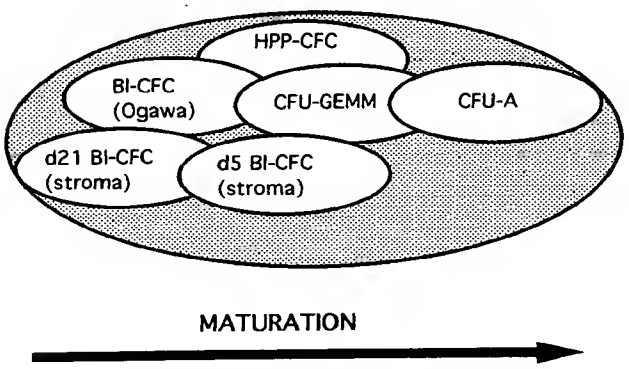


Fig. 2 Suggested relationship between primitive human progenitor cells measured directly by colony formation in vitro.

Secondary Colony Formation

This group includes assays for progenitor cells that do not themselves form colonies in semi-solid clonogenic assay systems. They are detected indirectly by measuring the numbers of clonogenic cells they produce.

Long-term Bone Marrow Culture (LTBMC) and Long-term Culture-initiating Cells (LTCIC). Long-term bone marrow cultures are the only available in vitro culture system that reproduces almost entirely the interrelationships between haemopoietic stem cells and the stromal cells of the microenvironment. Originally, they were developed for studies of murine haemopoiesis[24] but later were adapted for studies of human haemopoiesis.[25] They consist of two major components which are the haemopoietic cells and the stromal cells of the haemopoietic microenvironment. Aspirated bone marrow contains precursors of both the stromal component and haemopoietic cells and, consequently, the cultures can be set up quite simply by inoculating tissue culture flasks with bone marrow cells. Adherent stromal progenitors will adhere to the flasks and form a confluent stromal layer consisting of fibroblasts, fat cells, macrophages and some endothelial cells. Primitive haemopoietic progenitor cells become trapped within the developing stromal layer and undergo haemopoiesis.[26] Alternatively, long-term bone marrow cultures can be set up in two stages by growing the stromal layer to confluence and then adding haemopoietic cell suspensions. In this case, the primitive haemopoietic cells adhere to the stromal layer.[27] The two-stage system has the advantage that it can be used to detect primitive progenitors in sources, such as blood, that do not contain cells capable of producing the stromal layer. The progress of haemopoietic activity is monitored by measuring the numbers of clonogenic precursors, assayed in semi-solid culture systems for CFU-GM, that are released into the culture supernatant. This is done at weekly intervals when the cultures are fed by removing half of the supernatant (demi-depopulation) and replacing it with fresh medium and supplements.

The long-term bone marrow culture system does not provide any information about the numbers of primitive progenitors that are responsible for producing the CFU-GM. More recently, attempts have been made to convert it into a quantitative assay for primitive human haemopoietic progenitors that have become known as long-term culture-initiating cells (LTCIC). In support of the validity of this assay, Eaves et al[28] demonstrated a linear relationship between the numbers of cells used to seed a preformed stroma and the numbers of CFU-GM released into the supernatant 5 weeks later. However, this relationship is not absolute since the number of long-term culture-initiating cells in 5-week-old normal marrow cultures is only 25% of the original number. This was demonstrated by Udomsakdi et al[29] who subcultured long-term cultures of different ages and measured the rate of decline of long-term culture-initiating cells. Thus, the CFU-GM readout is, in reality, related to a fraction of the number of long-term culture-initiating cells in the original marrow sample. This alone does not invalidate the assay but it does mean that the absolute numbers of long-term culture-initiating cells in different sources of haemopoietic tissue cannot be compared unless the rate of decline is known for the test sample and a correction is used if it differs from that of normal long-term culture-initiating cells. For example, Udomsakdi et al[29] showed that the long-term culture-initiating cells in the blood of patients with chronic myeloid leukaemia (CML) declined to a far greater extent in long-term culture than normal long-term culture-initiating cells. This results in only 0.7% of the leukaemic progenitors being present at 5 weeks compared with the number present at the beginning of the culture period. This means that the readout for chronic myeloid leukaemic progenitors requires greater adjustment than the readout for normal marrow progenitors in order to obtain absolute values.

The 5-week incubation period is selected for two reasons. First, because the bone marrow used to initiate the cultures also contains CFU-GM and these more mature progenitors persist for some time. This has been evaluated by comparing cultures initiated with 4-hydroperoxycyclophosphamide (4-HC)-treated cells (i.e. devoid of CFU-GM) with cultures of untreated cells. At 5 weeks, the CFU-GM production by the two types of culture is the same indicating that all of the CFU-GM are by this time produced by the primitive progenitor cell population.[28] This is not to say that the primitive progenitor cells do not produce CFU-GM during the first 5 weeks of culture but simply that there is a background of pre-existing CFU-GM for this period of time. The second point concerns the perceived structure of the stem cell population. It is assumed that the stem cells that produce the CFU-GM at the beginning of the culture period are less primitive that those that produce CFU-GM later on in the culture period.

Long-term culture-initiating cells have been meas-

ured at limiting dilution to determine their frequency in samples of haemopoietic tissue.[30] Again, measurements are made at 5 weeks. It is likely that these measurements, which are made on individual cells, are influenced by the extinction of individual clones after the first one or two cell divisions as a result of the random occurrence of self-renewal and differentiation at the single stem cell level (Fig. 3).

Sutherland et al[30] established that the long-term culture-initiating cells amounted to 1 or 2 per 10 000 normal human bone marrow cells and constituted 1–2% of the CD34-positive cell population. Subcultures of the clones produced by individual long-term culture-initiating cells showed that they contained variable numbers (1–30) of CFU-GM and some of them contained, in addition, BFU-E. When CD34-positive cells were sorted on the basis of Thy-1 expression, the majority of the clonogenic cells were found to be Thy-1-negative whereas most of the long-term culture-initiating cells co-expressed Thy-1. Further phenotypic analysis of the CD34-positive Thy-1-positive subset of cells showed that they expressed low levels of CD38, CD71 and c-kit, were CD45RO-positive, CD45RA-negative and rhodamine 123 ^{dull}.[7] However, another study[31] suggests that the c-kit positive cell fraction contains the most primitive progenitor cells in human bone marrow.

The existence of long-term culture-initiating cells in human peripheral blood has been demonstrated and the cells have been characterised.[32] There are about 6 of these cells per ml of blood, which is 75-fold lower than the numbers of in vitro clonogenic progenitors in blood and 100-fold lower than the frequency of long-term culture-initiating cells in human bone marrow.

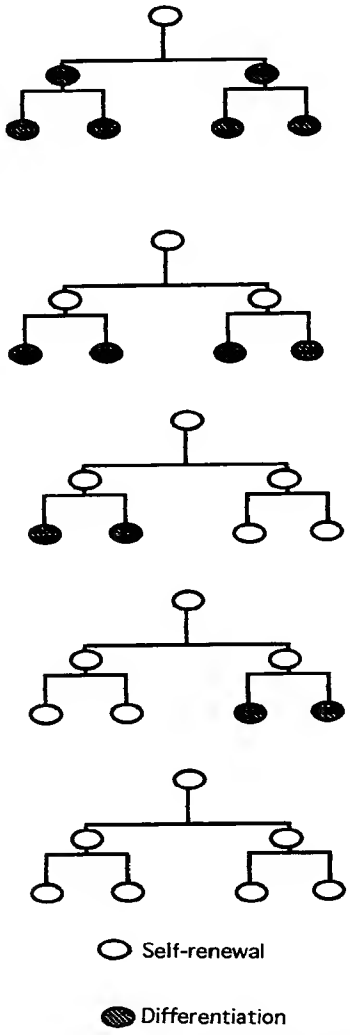


Fig. 3 The five possible results of the first two divisions by individual stem cells after Blackett (47). In steady state haemopoiesis, the probability of differentiation is 0.5 and after two divisions the average number of stem cells per clone will be unchanged. However, 12.5% of the clones will contain 4 stem cells, 25% will contain 2 stem cells and 62.5% will contain no stem cells.

Delta (Δ) Cultures of Stroma-adherent Progenitor Cells. A group of assay systems has become known as Δ assays because they measure the production by a single progenitor (apex of the triangle or Δ symbol) of multiple clonogenic progeny (base of the triangle). For example, cells bound to stroma by incubating them for 2 h with preformed stromal layers generate CFU-GM without the background of pre-existing CFU-GM which do not adhere to stroma.[22,23,33] For this assay, stroma plus adherent haemopoietic cells are incubated for one week and the CFU-GM in the supernatant are measured. The cells detected in the assay probably overlap with the long-term culture-initiating cells, which also adhere to stroma.[27] The stroma-adherent colony-forming cells (BI-CFC) (see above) may be the clonogenic equivalent of the stroma-adherent cells measured in the Δ assay.

Delta Assay of Plastic-adherent Progenitor Cells. This system is based on the finding that removal of plastic-adherent cells from murine bone marrow compromises its ability to repopulate the haemopoietic system when it is transplanted into irradiated recipients.[34–36] For this assay, human bone marrow mononuclear cells are incubated with tissue culture plastic for 2 h and then washed free of non-adherent cells. The adherent cells are incubated with medium, serum and growth factors for 1 week and the numbers of CFU-GM produced are enumerated using a semi-solid clonogenic assay.[37,38] The progenitors responsible for CFU-GM production are resistant to 5 fluorouracil treatment and express CD34 and Thy-1 (37,38, MYG-unpublished data).

Delta Cultures of Cells resistant to 4-hydroperoxycyclophosphamide. Smith et al[39] depleted CD34-positive human bone marrow mononuclear cells of CFU-GM by treating them with 4-hydroperoxycyclophosphamide (4-HC) and cultured them with interleukin-1 and interleukin-3 for 1 week before measuring the production of CFU-GM. They concluded that the 4-HC-resistant progenitors were primitive cells because they responded to interleukin-1, generated large numbers

of CFU-GM in culture and did not express lineage-related markers of haemopoietic cell differentiation.

This group of Δ assays and the long-term bone marrow culture system probably detect related and overlapping populations of early haemopoietic progenitor cells (Fig. 4).

Single Cell Cultures. For these experiments, cells are sorted or otherwise purified, and single cells are deposited into microwells for culture. The complicated procedures used for these studies make it difficult to obtain a quantitative measure of the frequency of the progenitors in a sample of haemopoietic tissue. This approach is most valuable for the qualitative characterisation of highly purified cells with defined phenotypic properties.

An example is provided by the work of Terstappen et al[40] who used multiparameter flow cytometry to isolate a CD34-positive CD38-negative cell population that constituted 1% of the total CD34-positive cell population. The CD38 antigen was expressed by all of the cells that expressed the lineage markers CD71 (erythroid), CD33 (myeloid) and CD10 (B lymphoid). Morphologically, the CD34-positive CD38-negative cells were homogeneous, slightly larger than lymphocytes, and had evenly dispersed chromatin, an irregularly shaped nucleus and scantly cytoplasm. The cells were sorted individually into wells and cultured without growth factors for 14 days. This step selects quiescent cells that can survive in the absence of growth factors. Then, a cocktail of recombinant human (rh) IL-3, rhIL-6, rhGM-CSF, rhG-CSF and rh erythropoietin was added to each well. Under these conditions, 25% of the CD34-positive CD38-negative cells produced colonies of 20–50 small loosely adherent translucent blast cells after a further 28 to 34 days. Very few such colonies grew from CD34-positive CD38-positive cells and none grew from CD34-negative cells.

Replating of individual first generation dispersed blast colonies revealed the presence of clonogenic progenitors within the clones and replating for four further generations was achieved. The first and second generation colonies were indistinguishable from one another. The third generation colonies contained more cells than the first and second generation colonies and showed signs of mixed myeloid and erythroid differentiation. Fourth generation colonies were either myeloid or erythroid. Replating in this manner was continued to the fifth generation and clonogenic progenitors were still found 140 days after sorting the original CD34-positive CD38-negative cells. This repetitive replating reflects the capacity of the original cells for self-renewal. In contrast the few colonies derived from CD34-positive CD38-positive cells had no replating ability. These experiments indicate that the complete sequence of haemopoietic cell development can be reproduced in vitro.

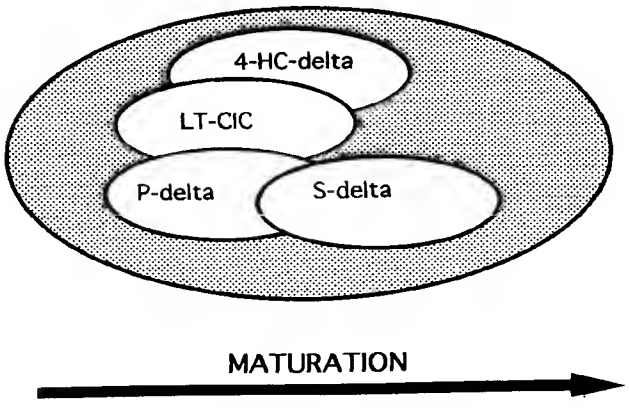


Fig. 4 Suggested relationship between primitive human progenitor cells measured indirectly by secondary colony formation in vitro.

Transplantation into Immunocompromised Mice

In the past, the transplantation of human bone marrow into mice in an attempt to produce a small animal model of human haemopoiesis has generally been unsuccessful. New approaches have focussed on the engraftment of human cells into immune deficient mice (reviewed in ref 41). The success of this strategy has been boosted by the development of two strains of mice, the SCID (severe combined immunodeficient) mice and bg/nu/xid (bnx) mice. Transplantation protocols vary and include intravenous, intraperitoneal and subcutaneous routes, the provision of a microenvironment of human origin and attempts to stimulate haemopoiesis by irradiating the recipient animals and by administering haemopoietic growth factors.

Kamel-Reid and Dick[42] injected human bone marrow cells intravenously into irradiated immune deficient mice who carried osmotic minipumps delivering human IL-3 and GM-CSF. After 14 days, human DNA was detected in the bone marrow and spleen of the transplanted animals and human progenitors of macrophage colony formation in vitro were detected for several weeks. It is possible that these macrophage colony-forming cells arose by differentiation from more primitive precursors because their numbers increased after transplantation and subsequently were maintained.

Human fetal bone fragments have been used to provide a haemopoietic microenvironment. When sorted CD34-positive Thy-1-positive fetal bone marrow cells were microinjected into fetal bone fragments and transplanted into the peritoneal cavities of SCID mice the bone grafts became vascularised and human haemopoiesis in the myeloid and B lymphoid compartments could be sustained for 3 months.[6] The same cell populations also produced long-term myelopoiesis and lymphopoiesis when co-cultured with stromal cells in vitro indicating that the SCIDhu model and cultures for long-term culture-initiating cells detect similar stem cell populations. Kyoizumi et al[43] performed similar experiments with

fetal bone marrow and obtained engraftment of myeloid, erythroid and lymphoid (B and T) lineages. The numbers of CFU-GM and BFU-E were low at 2 weeks but had increased 30-fold by 4 weeks after transplantation. Up to 2% of the peripheral blood cells of these SCIDhu mice were of human origin and the majority of them were B-cells.

Other experiments have introduced a human lymphopoietic microenvironment in order to study human lymphopoiesis in the SCIDhu model system. For example, human fetal CD34-positive Thy-1-positive cells differentiate into T-lymphocytes in human fetal thymus transplanted into SCID mice.[6,44,45] In addition, human fetal lymph node tissue in SCID mice supports B-lymphopoiesis.[44]

These murine models of human haemopoiesis provide, inter alia, vehicles for the investigation of human haemopoietic cell pathology and assays for the effects of chemotherapeutic agents and haemopoietic growth factors. The latter application has recently been demonstrated by Kyoizumi, Murray and Namikawa[46] who showed that G-CSF elicited expansion of neutrophilic granulocyte populations, IL-3 induced significant increases in eosinophilic granulocytes and BFU-E, IL-6 increased the numbers of CFU-GM and BFU-E and erythropoietin enhanced erythropoiesis. A major advantage of SCIDhu mice over in vitro cultures in this context is that the agents for testing will be influenced by mammalian physiology during administration.

Overview

There are many approaches to the investigation of human haemopoietic stem cells. All of them have contributed to our knowledge in this area of experimental haematology but, clearly, some of them are particularly well suited to specific applications. Obvious example are the use of the long-term bone marrow culture system for investigating regulatory interactions between stem cells and the haemopoietic microenvironment in vitro and the use of SCIDhu mice to test the effects of exogenous drugs and growth factors in a physiological setting.

Acknowledgement

The work was supported by The Leukaemia Research Fund of Great Britain.

References

1. Till J E, McCulloch E A, Siminovitch L. A stochastic model of stem cell proliferation, based on the growth of spleen colony forming cells. Proc Natl Acad Sci USA 1964; 51: 29–36.
2. Ogawa M, Porter P N, Nakahata T. Renewal and commitment to differentiation of hemopoietic stem cells: an interpretive review. Blood 1983; 61: 823–829.
3. Nicola N A, Johnson G R. The production of committed hemopoietic colony-forming cells from multipotential precursors in vitro. Blood 1982; 60: 1019–1029.
4. Wolf N S. The haemopoietic microenvironment. Clin Haematol 1979; 8: 469–500.
5. VanZant G, Goldwasser E. Competition between erythropoietin and colony-stimulating factor for target cells in mouse marrow. Blood 1979; 53: 946–965.
6. Baum C M, Weissman I L, Tsukamoto A S, Buckle A-M, Peault B. Isolation of a candidate human hematopoietic stem cell population. Proc Natl Acad Sci USA 1992; 89: 2804–2808.
7. Craig W, Kay R, Cutler R L, Lansdorp P M. Expression of Thy-1 on human hematopoietic progenitor cells. J Exp Med 1993; 177: 1331–1342.
8. Lansdorp P M, Dragowska W. Long-term erythropoiesis from constant numbers of $CD34^+$ cells in serum-free cultures initiated with highly purified progenitor cells from human bone marrow. J Exp Med 1992; 175: 1501–1509.
9. Lansdorp P M, Sutherland H J, Eaves C J. Selective expression of CD45 isoforms on functional subpopulations of hemopoietic cells from human bone marrow. J Exp Med 1990; 172: 363–366.
10. Sutherland H J, Eaves C J, Eaves A C, Dragowska W, Lansdorp P M. Characterisation and partial purification of human bone marrow cells capable of initiating long-term hematopoiesis in vitro. Blood 1989; 74: 1563–1570.
11. Udomsakdi C, Eaves C J, Sutherland H J, Lansdorp P M. Separation of functionally distinct subpopulations of primitive human hematopoietic cells using rhodamine-123. Exp Hematol 1991; 19: 338–342.
12. Gordon M Y, Barrett A J. Bone marrow disorders: The biological basis of clinical problems. Oxford: Blackwell Scientific Publications.
13. Carrow C E, Hangoc G, Broxmeyer H E. Human multipotent progenitor cells (CFU-GEMM) have extensive replating capacity for secondary CFU-GEMM: An effect enhanced by cord blood plasma. Blood 1993; 81: 942–949.
14. Ogawa M. Effects of hemopoietic growth factors on stem cells in vitro. Hematol Oncol Clin North Am 1989; 3: 453–464.
15. Bradley T R, Hodgson G S. Detection of primitive macrophage progenitor cells in mouse bone marrow. Blood 1979; 54: 1446–1450.
16. McNiece I K, Stewart D M, Deacon D S, et al. Detection of a human CFC with high proliferative potential. Blood 1989; 74: 609–612.
17. Srour E F, Brandt J E, Bridell R A, Grigsby S, Leemhuis T, Hoffman R. Long-term generation and expansion of human primitive hematopoietic progenitor cells in vitro. Blood 1993; 81: 661–669.
18. Holyoake T L, Freshney M G, Konwalinka G et al. Mixed colony formation in vitro by the heterogeneous compartment of multipotential progenitors in human bone marrow. Leukemia 1993; 7: 207–213.
19. Cohen G I, Canellos G P, Greenberger J S. In: Gale R P, Fox C F, Eds. In vitro quantitation of engraftment between purified populations of bone marrow haemopoietic stem cells and stromal cells. Biology of bone marrow transplantation. New York: Academic Press, 1980: 491–506.
20. Gordon M Y, Hibbin J A, Kearney L U, Gordon-Smith E C, Goldman J M. Colony formation by primitive haemopoietic progenitor cells in cocultures of bone marrow cells and stromal cells. Br J Haematol 1985; 60: 129–136.
21. Gordon M Y, Dowding C R, Riley G P, Greaves M F. Characterisation of stroma dependent blast colony-forming cells in human marrow. J Cell Physiol 1987; 130: 150–156.
22. Dowding C R, Gordon M Y. Physical, phenotypic and cytochemical characterisation of stroma-adherent blast colony-forming cells. Leukemia 1992; 6: 347–351.
23. Tucker D, Bol S, Kannourakis G. Characterisation of stroma-adherent colony-forming cells: A clonogenic assay for early hemopoietic cells? Exp Hematol 1993; 21: 469–474.
24. Dexter T M. Cell interactions in vitro. Cin Haematol 1979; 8: 453–468.
25. Gartner S, Kaplan H S. Long-term culture of human bone marrow cells. Proc Natl Acad Sci USA 1980; 77: 4756–4759.
26. Coulombel L, Eaves A C, Eaves C J. Enzymatic treatment of long-term human marrow culture reveals the preferential location of primitive hemopoietic progenitors in the adherent layer. Blood 1983; 62: 291–297.
27. Verfaillie C, Blakolmer K, McGlave P. Purified primitive hematopoietic progenitor cells with long-term in vitro repopulating ability adhere selectively to irradiated bone marrow stroma. J Exp Med 1990; 172: 509–520.

28. Eaves C J, Sutherland H S, Udomsakdi C, et al. The human hematopoietic stem cell in vitro and in vivo. Blood Cells 1992; 18: 301–307.
29. Udomsakdi C, Eaves C J, Swolin B, Reid D S, Barnett M J, Eaves A C. Rapid decline of chronic myeloid leukemia cells in long-term culture due to a defect at the leukemic stem cell level. Proc Natl Acad Sci USA 1992; 89: 6192–6196.
30. Sutherland H J, Lansdorp P M, Hemkelman D H, Eaves A C, Eaves C J. Functional characterization of individual hematopoietic stem cells cultured at limiting dilution on supportive marrow stromal layers. Proc Natl Acad Sci USA 1990; 87: 3584–3588.
31. Bridell R A, Broudy V C, Bruno E, Brandt J E, Srour E F, Hoffman R. Further phenotypic characterization and isolation of human hematopoietic progenitor cells using a monoclonal antibody to the c-kit receptor. Blood 1992; 79: 3159–3167.
32. Udomsakdi C, Lansdorp P M, Hogge D, Reid D S, Eaves A C, Eaves C J. Characterization of primitive hematopoietic cells in normal human peripheral blood. Blood 1992; 80: 2513–2521.
33. Gordon M Y, Hibbin J A, Dowding C, Gordon-Smith E C, Goldman J M. Separation of human blast progenitors from granulocytic, erythroid, megakaryocytic and mixed colony-forming cells by 'panning' on cultured marrow-derived stromal layers. Exp Hematol 1985; 13: 937–940.
34. Bearpark A D, Gordon M Y. Adhesive properties distinguish sub-populations of haemopoietic stem cells with different spleen colony-forming and marrow repopulating capacities. Bone Marrow Transplant 1989; 4: 625–628.
35. Kiefer F, Wagner E F, Keller G. Fractionation of mouse bone marrow by adherence separates primitive hematopoietic stem cells from in vitro colony-forming cells and spleen colony-forming cells. Blood 1991; 78: 2577–2582.
36. Kerk D K, Henry E A, Eaves A C, Eaves C J. Two classes of primitive pluripotent hemopoietic progenitor cells: separation by adherence. J Cell Physiol 1985; 125: 127–134.
37. Gordon M Y, Riley G P, Greaves M F. Plastic-adherent progenitor cells in human bone marrow. Exp Hematol 1987; 15: 772–778.
38. Gordon M Y, Clarke D, Healy L E. An in vitro model for the production of committed haemopoietic progenitor cells stimulated by exposure to single and combine recombinant growth factors. Bone Marrow Transplant 1989; 4: 353–358.
39. Smith C, Gasparetto C, Collins N, et al. Purification and partial characterisation of a human hematopoietic precursor population. Blood 1991; 77: 2122–2128.
40. Terstappen L W M M, Huang S, Safford M, Lansdorp P M, Loken M R. Sequential generations of hematopoietic colonies derived from single nonlineage-committed $CD34^+$ $CD38^-$ progenitor cells. Blood 1991; 77: 1218–1227.
41. Dick J E. Immune-deficient mice as models of normal and leukemic human hematopoiesis. Cancer Cells 1991; 3: 39–48.
42. Kamel-Reid S, Dick J E. Engraftment of immune-deficient mice with human hematopoietic stem cells. Science 1988; 242: 1706–1709.
43. Kyoizumi S, Baum C M, Kaneshima H, McCune J M, Yee E J, Namikawa R. Implantation and maintenance of functional human bone marrow in SCID-hu mice. Blood 1992; 78: 1704–1711.
44. McCune J M, Namikawa R, Kaneshima H, Schultz L D, Lieberman M, Weissman I L. The SCID-hu mouse: Murine model for the analysis of human hematolymphoid differentiation and function. Science 1988; 241: 1632–1639.
45. Peault B, Weissman I L, Baum C, McCune J M, Tsukomoto A. Lymphoid reconstitution of the human fetal thymus in SCID mice with $CD34^+$ precursor cells. J Exp Med 1991; 174: 1283–1286.
46. Kyoizumi S, Murray L S, Namikara R. Preclinical analysis of cytokine therapy in the SCID-hu mouse. Blood 1993; 81: 1479–1448.
47. Blackett N M. The kinetics of cell cloning assays: hemopoietic spleen colony growth. Int J Cell Cloning 1988; 6: 296–305.

BLOOD

The Journal of
The American Society of Hematology

VOL 78, NO 1 JULY 1, 1991

REVIEW: STRATTON LECTURE 1990

Clinical Implications of Positive and Negative Hematopoietic Stem Cell Regulators

By Malcolm A.S. Moore

THE FIRST PHASE of the development and clinical application of cytokines regulating hematopoiesis could be said to have been completed with the recent approval, for a variety of clinical uses, of erythropoietin, granulocyte colony-stimulating factor (G-CSF), and granulocyte-macrophage CSF (GM-CSF). The CSFs are proving of immediate practical benefit in cancer therapy in reducing the incidence of febrile neutropenia and shortening the duration of hospitalization in patients receiving chemotherapy alone or in conjunction with autologous marrow transplantation. Future clinical studies of G-CSF and GM-CSF will focus on whether the myeloprotection will permit chemotherapy dose and schedule intensification, which in turn could lead to improved cancer survival rates. We have now entered a second phase of clinical investigation using cytokines that act at earlier stages of hematopoietic and immune system development. Among this array of over 17 specific gene products that positively influence hematopoiesis (including 11 interleukins [ILs]), are cytokines that directly influence pluripotential stem cell proliferation and multilineage differentiation. To understand the mechanisms of action of these early acting cytokines, and to develop approaches for their effective clinical use, it is first necessary to develop a clearer understanding of the nature of the stem cell compartment.

CHARACTERIZATION OF PLURIPOTENTIAL STEM CELLS

The defining characteristic of a stem cell is its capacity for extensive self-renewal and retention of multilineage differentiation potential. It may be argued that true self-renewal is an unattainable goal and all daughter cells must differ in some respect, however slightly, from their parents. The acceptance of this concept leads to the view that the stem cell compartment is of necessity extremely heterogeneous and hierarchical. The ultimate derivation of hematopoietic stem cells remains, in all probability, the yolk sac blood islands, and all subsequent stem cell populations are established by a series of migration patterns with colonization of developing hematopoietic microenvironments culminating in the bone marrow as the major stem cell repository of the adult.[1,2]

If stem cells were capable of unlimited self-renewal, the clonal composition of the hematopoietic system would be stable over prolonged periods, even with fluctuating demand for mature hematopoietic cells. However, if self-renewal is limited a proportion of the stem cell pool could be drawn on to be active in the replacement of others that were exhausted in a "death by differentiation" process. Hematopoiesis would then be maintained by a succession of short-lived clones. Evidence for this clonal succession model, first proposed by Kay,[3] was provided by hemoglobin marker studies in W anemic mice injected in vitro with mixtures of fetal liver cells from two inbred mice strains.[4] Studies using retrovirally marked stem cells showed that few were simultaneously providing mature blood cells, supporting the concept that stem cell clones are used successively during the mouse life span and that the life span of some clones may approximate that of the mouse.[5,6] However, other studies analyzing chimerism of hemoglobin variants in either allophenic mice or recipients of transplanted marrow led to the conclusion that many, if not all, stem cells were simultaneously produced.[7,8] By combining retroviral marking and transplantation of chimeric hematopoietic tissue of allophenic mice marked by enzyme or hemoglobin variants, it has been shown that only a few pluripotent stem cells participate in the repopulation and long-term maintenance of engrafted hematopoietic and lymphoid tissue.[9] Furthermore, genotypic specificity can contribute to the relative contributions of marrow to early or late engraftment. Engraftment may be divided into an early phase of a few months during which multiple stem cell clones with transient and restricted development potential are active, and a later stage with a stable contribution from

From the James Ewing Laboratory of Developmental Hematopoiesis, Memorial Sloan-Kettering Cancer Center, New York, NY.

Submitted April 2, 1991; accepted April 15, 1991.

Supported by Public Health Service Grants CA-32516, CA-20194, and CA-23766 from the National Cancer Institute; and DK-42639 from the National Institutes of Health; American Cancer Society Grant CH-3K; and the Gar Reichman Fund of the Cancer Research Institute.

Presented as the Henry M. Stratton Memorial Lecture, American Society of Hematology, December 1990.

Address reprint requests to Malcolm A.S. Moore, D. Phil, Sloan-Kettering Institute for Cancer Research, 1275 York Ave, New York, NY 10021.

0006-4971/91/7801-0035$3.00/0

one or two pluripotent stem cells with long life spans.[10] This concept fits with the view that there are at least two subpopulations of stem cells differing in early repopulating ability versus long-term stem cell reconstitution and that genetic differences in the relative proportions of these subpopulations exist in different mouse strains. It should be noted that the decline in representation of one genotype over another in the differentiating hematopoietic cell population does not, a priori, indicate the demise of one stem cell population relative to another. Secondary transfer studies have shown that while one stem cell population can be clearly dominant in a primary recipient the suppressed population can be reactivated on marrow transfer to a secondary recipient.[9]

IN VIVO STEM CELL ASSAYS

The spleen colony forming (CFU-S) assay[11] was long considered the only true stem cell assay available and serial colony passage studies indicated that a minority of CFU-S were capable of extensive self-renewal.[12] Stochastic processes determining the probability of stem cell self-renewal versus differentiation were thought to account for this clonal variation in self-renewal potential. Subsequent studies showed that a hierarchic structure existed within the CFU-S compartment. Early appearing day 8 CFU-S lacked stem cell properties and overlapped with the committed progenitor populations detected by the in vitro colony-forming unit (CFU), granulocyte, erythroid, megakaryocyte, macrophage (GEMM), burst-forming unit erythroid (BFU-E), CFU granulocyte-macrophage (CFU-GM) assays.[13] In contrast, late-appearing day 12 to 13 CFU-S were capable of a degree of self-renewal in serial transplant studies. The inter-relationship of CFU-S with later progenitors and earlier cells detected in other bioassays is shown in Fig 1. Numerous investigators have purified D12 CFU-S,[14] and in one study a population of Thy-1 antigen and Ly6A-2 (Sca-1) positive and lineage marker negative marrow cells was highly enriched for D12 CFU-S and as few as 30 cells from this enriched population could rescue otherwise lethally irradiated mice.[15] Other studies have cast serious doubt that the majority of D12 CFU-S are representative of the early stem cell compartment as measured by long-term reconstitution of hematopoiesis. Table 1 outlines a number of markers that have been used to purify populations of early hematopoietic cells that could then be assayed for CFU-S and long-term reconstitution. Rhodamine-123 (Rh) is a powerful probe for the resolution and hierarchical ordering of transplantable hematopoietic stem cell populations. The relatively low number and/or inactivity of mitochondria in quiescent stem cell populations can be measured by the low retention of the supravital fluorochrome dye Rh. The majority of D8 and D12 CFU-S actively incorporate Rh (Rh-bright), but sustain hematopoiesis for only a short duration when transplanted into irradiated recipients. In contrast, Rh "dull" cells have an in vivo ability to sustain generation of D12 CFU-S and support long-term engraftment. The combination of selection of early hematopoietic cells based on expression of Ly6A (Sca-1) and

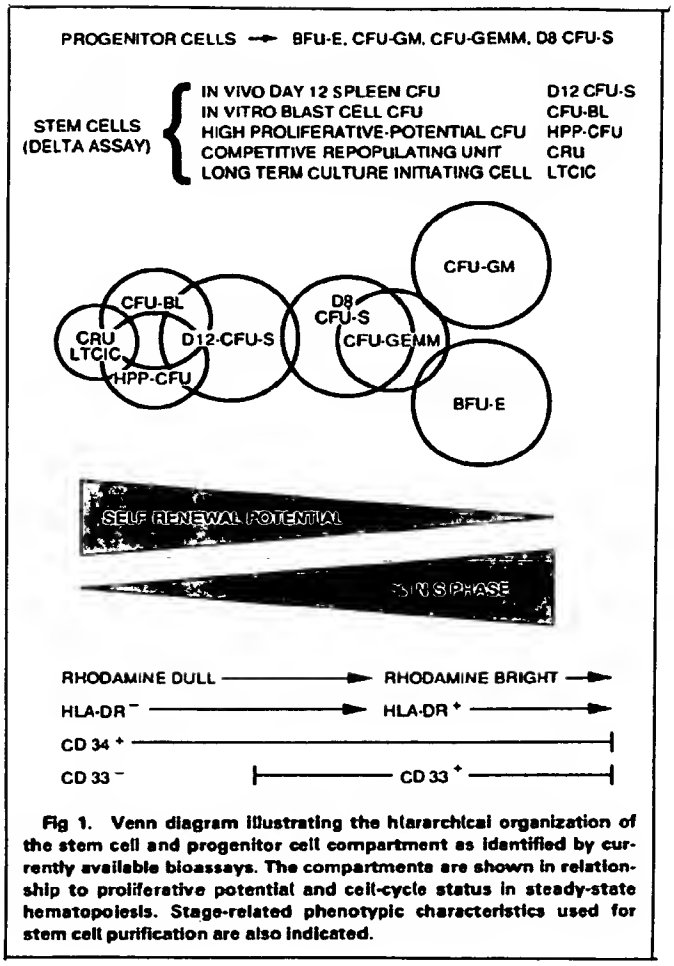


Fig 1. Venn diagram illustrating the hierarchical organization of the stem cell and progenitor cell compartment as identified by currently available bioassays. The compartments are shown in relationship to proliferative potential and cell-cycle status in steady-state hematopoiesis. Stage-related phenotypic characteristics used for stem cell purification are also indicated.

Rh "dull" confirmed that D12-13 CFU-S were predominantly in the Rh "bright" fraction, whereas the long-term reconstituting cells and cells capable of generating D12-13 CFU-S were selectively present in the Rh "dull" fraction.[24] Counterflow centrifugal elutriation that sorts cells on the basis of both size and density provided a simple procedure for selective separation of early hematopoietic cell populations in murine marrow.[20] Durable long-term engraftment of irradiated mice was obtained with slowly sedimenting fractions nearly completely depleted of committed progenitors and D12 CFU-S (<0.25% of total D12 CFU-S), whereas fractions containing 95% of total CFU-S were ineffective in supporting late (day 60) reconstitution.[20]

IN VITRO STEM CELL ASSAYS

Long-term culture initiating cells. Sustained in vitro hematopoiesis for weeks or months can be attained in culture systems in which primitive hematopoietic stem cells are maintained in intimate interrelationship with a layer of marrow-derived stromal cells. These long-term bone marrow cultures (LTBMC) were first developed to support murine stem cell (CFU-S) replication[25] and were subse-

Table 1. Properties of HSC Used for Stem Cell Enrichment

Murine	Reference	Human	Reference
5-FU-resistant	16,17	4-HC rasistant	35,80,82,83
Slow sedimantation rate	20,21,20	CD34-positiva	36,38-42,81,82,83
Light buoyant density	21,22	CD45 R0 isoform-positiva	42
Low Hoechst 33342 dye uptake	18	Lineage marker-negative (eg, CD33)	33,35,36,38-42,82,83
Low Rh-123 uptake	19,23,24,30	HLA-DR-negative	33,35,36,38-41
High clasa 1 MHC (K/D)	16,23	Low forward and right angle light scatter	38-41,82
Thy-1 (low)	16		
Ly-6 A.2 (Sca-1)-positive	15,23,24		
Lineaga marker-negativa	15		

quently adapted to sustain long-term hematopoiesis with marrow from other species, including humans.[26,27]

In vitro, long-term culture-initiating cells can be assayed by their ability to sustain prolonged (weeks) hematopoiesis and progenitor cell generation when inoculated onto preformed irradiated marrow stroma. In murine systems, 5-fluorouracil (5-FU) treatment enriches for cells with the capacity to initiate long-term hematopoietic activity on preformed stroma while profoundly depleting D12 CFU-S.[28] Indeed, highly purified D12 CFU-S are unable to sustain long-term hematopoiesis on stromal layers.[29] Rh "bright" marrow populations containing the majority of D8 and D12 CFU-S and in vitro clonogenic progenitors sustained hematopoiesis only in the early stages when inoculated onto irradiated stroma. Long-term reconstitution in vitro was obtained only with Rh "dull" cells.[30]

We first proposed that human pluripotential stem cells lacked HLA-DR antigen by showing that anti–HLA-DR antisera plus complement did not ablate the capacity of human bone marrow to sustain long-term production of HLA-DR^{+ve} CFU-GM and BFU-E when inoculated onto preformed marrow stroma.[31] This finding was confirmed by Keating et al[32] and Andrews et al[33]; however, others have provided evidence for DR expression even on apparently early stem cells.[34] Bruhl et al[35] showed that 4-hydroperoxycyclophosphamide (4HC)-resistant stem cells (pre–CFU-GM) that could support long-term progenitor cell production on irradiated stroma were HLA-DR$^-$, DP$^-$, DQ$^-$, and DY$^-$. Brandt et al[36] also characterized the human marrow blast colony forming cell as CD34$^+$, HLA-DR$^-$. However, Caux et al[37] reported that CD34$^+$ cells panned from cord blood coexpressed DR and DP and the majority also expressed DQ. It is possible that class II antigen expression is related to the activation state of the stem cell and that low to absent HL-DR expression is a feature of quiescent cells in deep Go. Cytokine activation, for example, by IL-1, IL-6, IL-3, GM-CSF, or interferon (IFN) may upregulate DR expression. Thus, in the experimental design of various studies that demonstrate HLA-DR expression on early stem cells, there is frequently sufficient opportunity for cells to be activated by endogenous cytokine production by accessory cells. If expression is proliferation dependent, the possible HLA-DR$^+$ status of cord blood stem cells could correlate with the active cell cycle status of the stem cells at this critical stage of development.

In recent studies the combination of negative selection by depletion of HLA-DR$^+$ cells and other lineage-positive cells (eg CD33, CD15, CD77) with positive selection for CD34-positive cells exhibiting low right angle and low forward light scatter properties has permitted the enrichment of a cell population comprising 1.4×10^{-6} to 1.4×10^{-5} cells of the marrow possessing long-term culture-initiating properties[38-42] (Table 1). The CD34$^+$, HLA-DR$^+$ cells initiate early colony-forming cell (CFC) generation on preformed stroma but with early exhaustion, the CD34$^+$, DR$^-$ cells generated CFC for at least 12 weeks. Cloning of two color-sorted cells into culture wells containing irradiated bone marrow stroma showed that 3% to 4% of CD34$^+$, Lin$^-$ cells generated CFC after 4 weeks.[41]

Gordon et al[43] identified a population of early human hematopoietic cells characterized by an ability to specifically adhere to preformed irradiated marrow stroma within 2 hours. These adherent cells had stem cell features in that they formed blast cell colonies, had self-renewal potential, and generated multipotential and lineage-committed colony-forming cells. Subsequent studies showed that the cell type specifically adhering to the stroma was CD34$^+$, HLA-DR$^-$, Lin$^-$, and following this biologic "panning" the specifically adhered cells reconstituted long-term hematopoiesis.[40]

Blast colony assay (CFU-B1). An assay based on enumeration of small colonies of morphologically undifferentiated blast cells (CFU-B1) detects an early hematopoietic cell type with high secondary recloning capacity, self-renewal potential, and the capacity to generate various multilineage committed progeny[36,44,45] (Fig 1). In both murine and human systems, IL-3 provides a permissive environment for the proliferation of these early progenitors in culture but does not initiate the transition from Go to active cell cycle. In prolonged culture in the presence of low concentrations of IL-3, CFU-B1 preferentially survive relative to later progenitor populations. In spleen and bone marrow cultures established from 5-FU–treated mice, IL-3 and IL-6 acted synergistically to support CFU-BL[46,47] and the time required for initiation of blast colony formation was significantly reduced in culture containing both ILs. The combination of IL-3 and IL-6 acted synergistically to yield multilineage, oligolineage, and unlineage colonies. IL-1 also acted as a synergistic factor in this assay but the effect was less than that obtained with IL-6. In a human CFU-B1 assay, CD34$^+$ marrow cells were established for a prolonged period before addition of IL-3, IL-6, or IL-1 or a combination of these factors, and blast cell colony formation assayed after 3

to 4 weeks of culture. Accelerated blast cell colony formation was observed after stimulation with IL-6 plus IL-3, but not with IL-1 plus IL-3.[44]

High proliferative potential colony forming cell (HPP-CFU) assay. HPP-CFU in murine bone marrow were first characterized as resistant to in vivo 5-FU treatment and closely identified with cells with long-term reconstituting capacity after bone marrow transplantation[48] (Fig 1). In contrast to CFU-S that are reduced by 98% in 24 to 48 hours after 5-FU treatment, HPP-CFU are resistant, indeed are enriched by over 10-fold as a result of depletion of other marrow elements.[28] The HPP-CFU assay detects colonies of greater than 0.5 mm diameter at 10 to 12 days of culture, containing greater than 50,000 cells per clone. The HPP-CFU population is clearly heterogeneous (Fig 2). Hierarchical subpopulations can be identified based on kinetics of regeneration after 5-FU treatment and in specific cellular requirements for cytokine signals.[49-51] Synergism between specific CSF species, including IL-3, leads to development of large colonies (HPP-CFU-2) that are developmentally derived from cells at a progenitor/CFU-S stage.[51] Precursors of these cells (HPP-CFU-1) are detected in cultures stimulated by a CSF species plus a synergistic activity that lacks direct colony-stimulating capacity.[48-56] Synergistic activities were identified in a variety of cell– and cell-line–derived conditioned medias, including that of the human

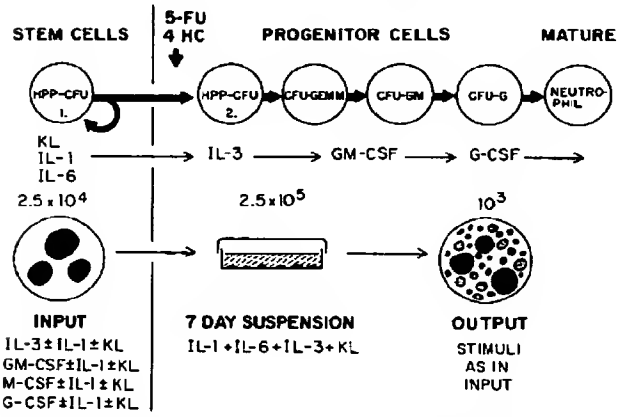


Fig 2. The development of neutrophils from stem cells identified by the high proliferative potential colony assay (HPP-CFU-1) via pluripotent, oligopotent, and unipotent progenitor cells detected in specific clonogenic assays. The interaction of cells at each step in this pathway with specific cytokines is indicated. The delta assay (or secondary recloning assay) involves the cloning of murine bone marrow 24 hours post in vivo 5-FU treatment, or in humans, post in vitro treatment of CD34⁺ marrow cells with 4-hydroperoxycyclophosphamide (4HC). The input or primary cloning efficiency is determined in the presence of the indicated cytokine combinations. A 7-day suspension culture of stem cell enriched marrow with cytokine combinations is followed by recloning at lower cell densities (10^2 to 10^3 cells per milliliter) with single CSF species for secondary CFU-GM, and with cytokine combinations for secondary HPP-CFU determinations. Amplification or self-renewal of HPP-CFU is the ratio of 2° HPP-CFU output over 1° HPP-CFU input. The generation of committed progenitors, eg, CFU-GM, from HPP-CFU is the ratio of total secondary CFU-GM responsive to a single CSF species over the input numbers of HPP-CFU responsive to dual or multiple cytokine signals.

bladder cancer line 5637, first identified as a source of G-CSF.[49-56] A synergistic activity termed hemopoietin-1[53] derived from this cell line was subsequently shown to be interleukin-1.[49,50,54] IL-1, both α and β forms, synergize with G-CSF, M-CSF, GM-CSF, and IL-3 in development of HPP colonies, and colony morphology is determined by the CSF species used.[49,55,56] IL-1 also synergizes with IL-3 and erythropoietin in the development of large erythroid bursts and multilineage colonies,[55,56] and with IL-3 and IL-5 in the generation of eosinophils.[56,57]

Synergism between IL-6 and M-CSF or IL-3 is also seen in the HPP-assay, and supradditive interactions take place between IL-1, IL-6, and G-CSF, M-CSF or GM-CSF.[55] These supradditive interactions of IL-1 and IL-6 disprove the notion that IL-1 acts indirectly via induction of IL-6 production via accessory cells.[47] Limiting dilution analysis, HPP purification, and accessory cell depletion studies and cultures undertaken in serum-free conditions all point to the direct interaction of IL-1 with early hematopoietic cell populations. Reports of IL-1 potentiation of hematopoietic colonies developing from progenitor cells are, however, likely to be due to secondary induction of CSF species by accessory cells because there is no evidence that IL-1 directly influences proliferation and differentiation of accessory cell-depleted populations of CFU-GM or BFU-E. Cell sorting on the basis of Rh-123 fluorescence in conjunction with immunomagnetic selection has shown that IL-1 plus IL-3 plus M-CSF responsive HPPs and IL-1 plus IL-3 responsive cells copurify in the Rh-dull fraction that has in vivo long-term reconstituting capacity.[58] In contrast, HPP-CFC subsets in the Rh-123 bright fraction have a more restricted growth factor requirement and are predominantly IL-3 plus M-CSF responsive with significant numbers of HPP responsive to IL-3 alone and M-CSF alone. These latter (HPP-CFC-2) are more closely related to the committed progenitor compartment (Fig 2).

The 5637 cell line conditioned media was a consistently better source of "synergistic activity" for HPP-CFU than were the combinations of IL-1 or IL-6 and CSFs, suggesting the existence of yet other stem cell-active factors. Subsequent studies showed that 5637 constitutively produced an activity termed HILDA that stimulated proliferation of a murine IL-3–dependent cell line and was shown to be identical to leukemia inhibitory activity (LIF) that promotes the proliferation of embryonic stem cells and may stimulate early hematopoietic cells in the CFU-B1 assay.[59-61] A further synergistic activity has been identified as *kit* ligand (KL).[62-67]

KL in hematopoietic stem cell and progenitor assays. The c-*kit* proto-oncogene encodes a transmembrane tyrosine kinase receptor for an initially unidentified ligand and is allelic with the murine white spotted locus (W).[62] W mutations affect melanogenesis, gametogenesis, and hematopoiesis during development and adult life.[63] The effects of mutations at the W locus involve the erythroid lineage, resulting in macrocytic anemia, and the mast cell lineage resulting in complete absence of connective tissue and mucosal mast cells. At the pluripotential stem cell level, W mutations result in absence of normal spleen colony-

forming (CFU-S) cells in marrow. These hematopoietic defects are completely resolved by transplantation of +/+ litter mate control bone marrow stem cells, indicating that the defect is intrinsic to the stem cells and their progeny and not to the hematopoietic microenvironment. Mutations at the Steel (Sl) locus also result in a spectrum of defects in melanogenesis, gametogenesis, and hematopoiesis.[63] The latter are not corrected by stem cell transplants and require engraftment of spleen or marrow stromal tissue. In vitro we showed that hematopoiesis was defective in long-term marrow cultures of both WW^v and Sl/Sl^d marrow but that coculture of WW^v marrow stroma with Sl/Sl^d hematopoietic cell restored fully functional long-term hematopoiesis with reconstitution of stem cell (CFU-S) self-renewal.[64]

Using different approaches the ligand for c-*kit* was identified by three independent groups. Mast cell proliferation provided one assay for the KL. Normal mast cells proliferate in vitro in response to IL-3 and IL-4. These factors are uniquely T-cell derived and are distinct from an additional mast cell growth factor derived from 3T3 fibroblasts.[65,66] Normal mast cells, but not mast cells from mice homozygous for a number of W alleles, proliferated in coculture with 3T3 cells in the absence of IL-3. This result suggested that the ligand for the c-*kit* receptor is produced by mature fibroblasts. Nocka et al[65] and Huang et al[66] developed a short-term mast cell proliferation assay that enabled the purification of a fibroblast activity, which, in the absence of IL-3, supported the proliferation of normal marrow-derived and peritoneal mast cells, but not WW^v mast cells. Williams et al[67-69] also used mast cell growth as an assay and named their factor mast cell growth factor (MGF). Zsebo et al,[70-72] using an assay based on proliferation of HPP-CFC in 5-FU–treated marrow, identified a KL from a rat liver cell line and termed it stem cell factor (SCF). To add to the current nomenclature confusion, the term Steel factor[73] has been proposed, reflecting the derangement or deletion of the gene product in Sl mutant mice. For purposes of clarity the term kit ligand (KL) will be used in this report.

The sequences for soluble rat, mouse, and human forms of KL have been reported,[70-72] as has the sequence for an alternative cell-bound form.[66] The natural protein is heavily N- and O-glycosylated and probably exists as a dimer. Glycosylation is not essential for activity because *Escherichia coli*-derived material is highly active.[70] The gene for KL maps at the Sl locus, and analysis of cell lines from Sl homozygous mice showed that frank deletions of KL as well as truncated forms of the gene may be observed.[66,69,70] KL exists as a cell surface protein and the notion that it might not be freely diffusible would be compatible with the results of transplantation and aggregation chimera experiments that indicate that the products of the Sl locus are not long range.[74] It is possible that small amounts of KL could be released in a soluble form either by protease cleavage of a membrane-bound form or by alternative splicing to generate a form no longer anchored to the cell membrane.[66]

The biologic actions of KL include stimulation of mast cell proliferation[65,66,68,69]; synergy with erythropoietin in stimulation of BFU-E and CFU-GEMM[65,66,68,71,75]; synergy with IL-3, G-CSF, and GM-CSF in stimulation of CFU-GM of human[76] and murine origin[71,75]; synergy with IL-7 in development of colonies of pre-B cells[71]; and synergy with IL-6, IL-1, or IL-3 in development of HPP-CFC.[70] Some species restrictions exist, and while rat and mouse KL will stimulate human hematopoietic cells, human KL is 800-fold less active on mouse cells.[71] In vivo, pharmacologic doses of KL markedly reduced the severity of the macrocytic anemic of Sl/Sl^d mice, inducing a leukocytosis and thrombocytosis with stimulation of large numbers of mast cells at the injection site.[70]

We have used murine KL (recombinant murine MGF [rm MGF]) in normal murine bone marrow cultures and observed very few myeloid colonies stimulated with KL alone, but a substantial increase in both colony number and size was seen with combinations of KL and G-CSF, GM-CSF, and IL-3, but not with M-CSF.[77] In HPP-CFC assays using marrow 24 hours post 5-FU treatment, increasing colony stimulation was seen with combinations of cytokines. KL plus either G-CSF, GM-CSF, IL-3, IL-7, or IL-6 was effective and combinations of three or four factors were even more effective in stimulating HPP-CFC. CSFs or IL-3 combined with IL-1, IL-6, and KL were maximally effective. Figure 3 shows HPP-CFC stimulated by cytokine combinations in cultures of 4-day post 5-FU murine marrow. In dual cytokine combinations, IL-1 plus GM-CSF or IL-3 stimulated comparable numbers of HPP-CFC, as did KL plus

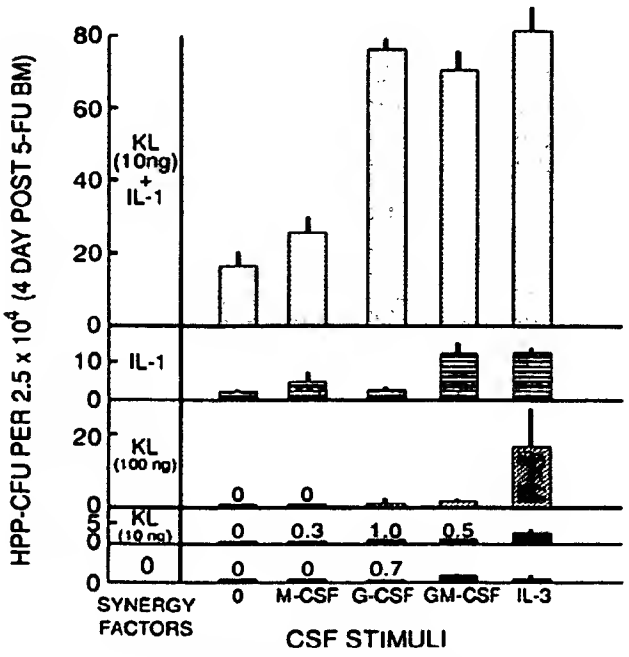


Fig 3. Synergism between recombinant human (rh) IL-1β (100 U/mL), rmKL (10 to 100 ng/mL), and rhM-CSF, rmGM-CSF, rhG-CSF, and rmIL-3 (all at 1,000 U/mL) in the HPP-CFU assay. Four-day post-5-FU murine bone marrow was cultured in 60-mm Petri dishes with a 2 mL 0.5% agarose underlayer containing cytokines, overlayed with 1 mL of 0.38% agarose containing 2.5 × 10^4 marrow cells. Following a 12-day incubation under reduced oxygen conditions, cultures were scored for colonies of greater than 0.5 mm diameter.

IL-1 or KL plus IL-3, but three factor combinations of IL-1 plus KL and either G-CSF, GM-CSF, or IL-3 were maximally effective.[77]

Delta or secondary CFU assay for early hematopoietic cells: Murine studies. The delta assay involves the short-term (7-day) suspension culture of bone marrow depleted of committed progenitors and enriched for early stem cells in the presence of various cytokine combinations (Fig 2). The ability of these cytokines to promote survival, recruitment, differentiation, and expansion of stem cells and progenitor cells is measured in a secondary clonogenic assay. In the murine system, the marrow is obtained 24 hours post 5-FU (Fig 2) or involves separation of early murine stem cells based on the criteria in Table 1. 5-FU–resistant stem cells are assayed in a primary HPP-CFC assay with multiple cytokine stimuli as well as in conventional CFU-GM assays with single CSF stimuli. After suspension culture secondary HPP-CFC and CFU-GM assays are performed. Three parameters are routinely measured. First is the amplification of lineage-restricted progenitors determined by the total CFU-GM responsive to a single CSF species (eg, G-CSF) in the primary culture (input) divided into total number of secondary CFU-GM responsive to the same CSF species in the secondary culture (output). Second is the ratio of HPP-CFC input divided into the total number of CFU-GM progenitors in the secondary assay. Because CFU-GM are presumed to derive from earlier precursors, ie, HPP-CFC, this ratio gives the indication of stem cell to progenitor cell differentiation. Finally, the ratio of HPP-CFC input divided into the total number of secondary HPP-CFC is determined. This parameter is the best measure of stem cell self-renewal, particularly if the HPP-CFC stimulus in the primary and secondary cultures is a combination of IL-1, IL-3, and KL.

In earlier studies (before the availability of KL), varying degrees of expansion in the numbers of CFU-GM responsive to single CSF species, and in HPP-CFC-1 and 2, were seen when IL-1 was combined with M-CSF (20- to 30-fold increases), with G-CSF (50- to 100-fold increases), with GM-CSF (100- to 500-fold increases), and with IL-3 (50- to 200-fold increases).[49,50,55,56] IL-3 and GM-CSF produced a limited degree of progenitor cell expansion whereas M-CSF and G-CSF did not. IL-6 was less effective than IL-1 in synergizing with M-CSF, GM-CSF, or G-CSF but was equally effective in synergizing with IL-3. IL-1 plus IL-6 showed additive or supradditive interactions with the three CSFs and IL-3. When KL (rm MGF-Immunex, Seattle, WA) was present in the suspension culture phase only a minor amplification of progenitor cell production occurred (Fig 4) but when combined with GM-CSF, IL-3, or IL-1, 200- to 800-fold amplification occurred. The combination of IL-1, KL, and either GM-CSF or IL-3 was even more effective in amplifying progenitors, and the four factor combination of IL-1 + KL + IL-6 with either IL-3 or GM-CSF produced up to 2,500-fold increases in progenitor cells. Calculations of progenitor cell generation based on CFU-GM output/HPP-CFC input showed that three factor combinations (IL-1 + KL + IL-3 or CSFs) generated ratios of 6,000 to 10,000, and four factor combinations (including

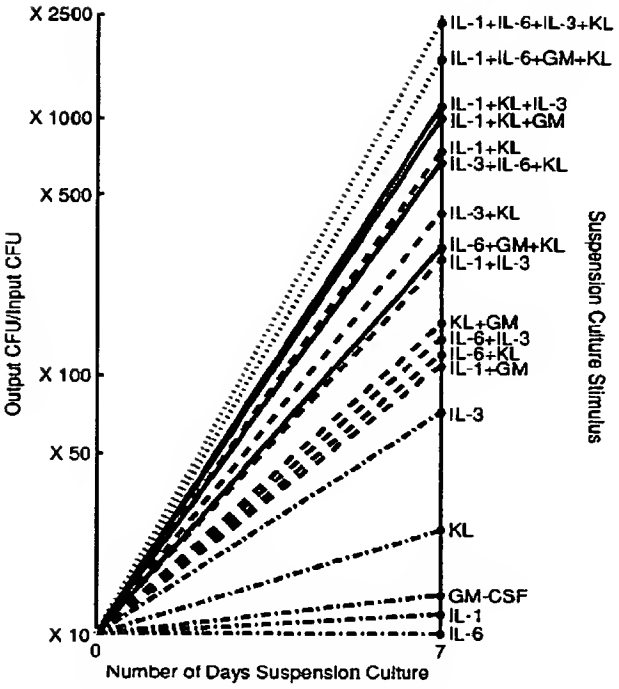


Fig 4. Secondary CFU-GM or delta assay showing the fold increase of GM-CSF–responsive CFU-GM in a 7-day suspension culture of 24-hour post 5-FU murine bone marrow. Marrow cells (2.5 × 10^5/mL) were cultured for 7 days with the cytokine combinations indicated and recovered cells recloned in a GM-CSF–stimulated colony assay. The fold increase is the ratio of the number of CFU-GM recovered in the secondary clonogenic assay over the input number of CFU-GM determined in the primary clonogenic assay with GM-CSF. rmKL was used as 20 ng/mL, rhIL-6 at 50 ng/mL, rhIL-1β at 100 U/mL, and rhGM-CSF or rmIL-3 at 1,000 U/mL.

IL-6) generated ratios of 8,000 to 15,000. As measure of self-renewal the generation of secondary HPP-CFC-1 as a ratio of HPP-CFC input reached values of 50 to 700 with two factor combinations of KL with IL-1, IL-3, or CSFs and 700 to 1,300 with three factor combinations of IL-1 + KL with IL-6, IL-3, or CSFs.

Based on the total differentiating cells produced in a 7-day culture of enriched HPP-CFC exposed to a combination of IL-1 plus IL-3 plus KL, Fig 5 illustrates the dramatic proliferation obtained. This includes a self-renewal component measured by secondary HPP-CFC-1 generation, a progenitor cell production measured by low proliferative potential CFU-GM, and morphologically identifiable differentiating myeloid cells. The cell population doubling time required to generate these cells from a single precursor reaches the limits of known mammalian cell proliferation rates. If this proliferation was sustained by an earlier even more infrequent cell than the HPP-CFC, an even shorter population doubling time would be required. The amplification of HPP-CFC in this short-term culture is unlikely to be reflected in a comparable expansion in long-term reconstituting cells, and the majority of HPP-CFC generated are more likely to be representative of later stages within the stem cell hierarchy. Assay of D12 CFU-S also showed an

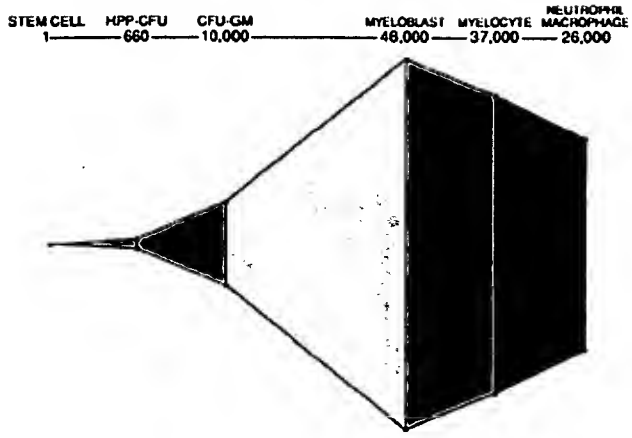


Fig 5. Amplification of hematopoiesis in cultures of 24 hour post 5-FU bone marrow cultured for 7 days in suspension in the presence of IL-1 + IL-3 + KL. Cells, 10^4, (after subtraction of granulocytes and lymphocytes) and containing 2.5% HPP-CFU responsive to IL-1 + IL-3 + KL in primary clonogenic assay, were incubated in suspension and the total cells and HPP-CFU responsive to IL-1 + IL-3 + KL, or CFU-GM responsive to rmGM-CSF were determined after 7 days in secondary clonogenic assays. The calculations are based on the ratio of output cells to input HPP-CFU.

absolute increase in numbers after 7 days of suspension culture with IL-1 plus IL-3 or KL.[77,78] Other investigators have shown that in similar suspension cultures, precursors of CFU-GEMM (possibly long-term reconstituting stem cells) also amplified in the presence of IL-1 plus IL-3 but not with IL-6 and IL-3 or GM-CSF combinations.[79]

Delta or secondary CFU assay for early hematopoietic cells: Human studies. In humans, 4-HC treatment of bone marrow has been shown to deplete the majority of progenitors capable of responding directly to GM-CSF by in vitro colony formation while preserving stem cells capable of hematopoietic reconstitution in the context of bone marrow transplantation.[80] In primate transplantation studies, $CD34^+$ selection also enriched for marrow cells capable of long-term reconstitution.[81] Following combined 4-HC treatment and selection of $CD34^+$ cells by immunocytoadherence, primary colony formation in response to G-CSF or GM-CSF was extremely low. However, 7 days of suspension culture followed by secondary recloning with GM-CSF showed that exposure of treated marrow cells for 7 days in suspension to combination of IL-1 and IL-3 consistently generated the highest numbers of secondary CFU-GM. IL-3 with IL-6 was no less effective than IL-3 alone and other cytokine combinations were significantly less effective.[82,93] Secondary colony formation in this assay was maximally stimulated by combinations of IL-1 and KL, KL and IL-3, and combination of all three cytokines was most effective in amplifying progenitor cell generation.

This secondary recloning/delta assay has been used in a number of experimental settings to determine whether it can provide predictive information to the quality of cells in a transplant or chemotherapy regeneration situation. In primates treated with 5-FU, conventional CFU-GM assay of bone marrow with GM-CSF stimulation showed prolonged suppression with recovery in 21 days, whereas populations of marrow cells with high IL-1 + IL-3 induced secondary CFU values were substantially elevated at 7 day post chemotherapy and peaked at 14 days.[82] A similar pattern of pre-CFU recovery was seen in chemotherapy myelosuppressed primates receiving in vivo IL-3 therapy.[82]

Purification of human pre-CFU populations was also achieved using a combination of soybean agglutination to remove the majority of T, B, and natural killer (NK) cells, and erythroid and mature myeloid cells. Depletion of $CD33^+$ and $MO1^+$ myeloid cells was accomplished by immunocytoadherence and complement-mediated cytotoxicity. This procedure removes the majority of CFU-GM directly responsive to CSF.[82] Enrichment for $CD34^+$ cells by positive immunocytoadherence yielded populations of greater than 90% $CD34,^+$ $CD33^-$ by FACS fluorescence-activated cell sorter (FACS) analysis with low 90° angle light scatter and low to medium forward angle scatter.[82] This population yielded substantial numbers of secondary CFU-GM after IL-1 plus IL-3 suspension culture. In HPP-CFC assays with IL-1 plus IL-3 this population also yielded plating efficiencies 600-fold greater than unfractionated cells, and when inoculated onto preformed irradiated marrow stroma generated substantial numbers of CFU-GM at 4 weeks.[82]

IN VIVO STUDIES WITH IL-1 ALONE OR IN COMBINATION WITH CSFs

The in vitro studies indicate that IL-1 can prime early stem cells to respond to CSFs and at the same time can induce the production of CSFs and other cytokines by an action on stromal cells.[37,84] This finding suggested the potential of IL-1 in vivo therapy to counteract myelosuppression. We first observed that IL-1 given within 6 to 18 hours of treatment with 150 mg/kg 5-FU in mice reduced the severity of subsequent neutropenia and accelerated recovery of neutrophils, and CFU-S, HPP-CFU, and CFU-GM in marrow and spleen.[49,55,56] IL-1 was more effective in accelerating neutrophil recovery than was G-CSF alone, but the combination of IL-1 and G-CSF produced additive to supraadditive stimulation of neutrophil recovery. IL-1 administered after treatment with cyclophosphamide (CY) also accelerates myeloid regeneration and platelet production.[85] Protection against alkylating agents and irradiation-induced myelosuppression was seen when IL-1 was given 20 to 24 hours before these myelosuppressive insults.[86,87] The protective effect in these models may involve activation of enzyme pathways that confer protection in stem cell populations, eg, aldehyde dehydrogenase that inactivates bioactive CY metabolites, or manganous superoxide dismutase that protects against damage from reactive oxygen species.

Protection against myelosuppression following 5-FU was not seen when IL-1 was administered 24 hours before chemotherapy (Fig 6). Indeed, doses of 0.1 to 0.2 μg per mouse of recombinant human IL-1 α or β given before 5-FU increased mortality and the severity of subsequent neutropenia. We observed that low doses (0.01 μg/mouse) of IL-1 administered before chemotherapy did not change the kinetics of subsequent neutrophil recovery (Fig 6), but

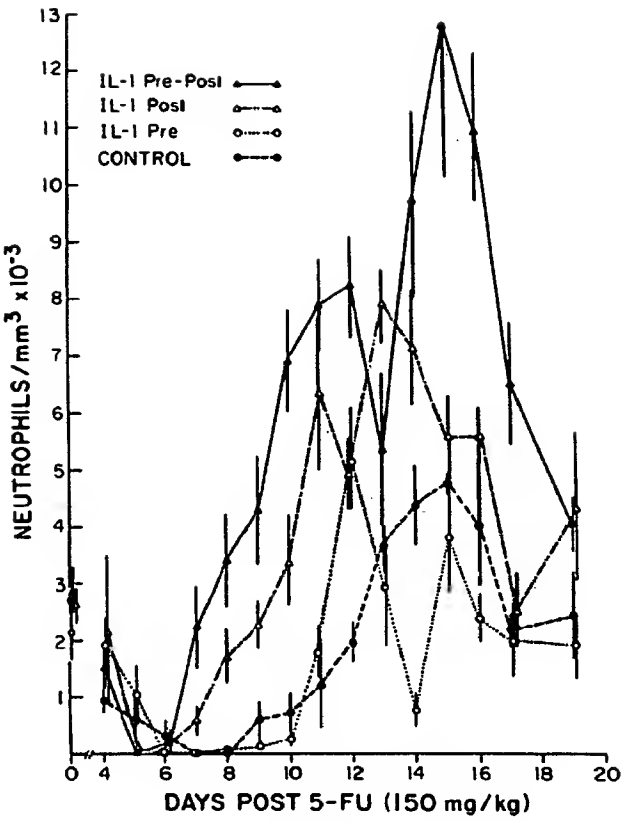


Fig 6. Neutrophil counts in Balb/c mice receiving 5-FU (150 mg/kg) as a single dose intravenously (IV) on day 0 (control, ●) or pretreated with 0.01 μg rhIL-1β per mouse IV 24 hours before chemotherapy (○). Animals were also treated with rhIL-1 intra-peritoneal (i.p.) beginning 12 hours post chemotherapy at a dose of 0.2 μg/mouse and repeated at 12-hour intervals for 4 days (△). A fourth group of mice received IL-1 (0.01 μg) 24 hours prechemotherapy and a 4-day cycle of IL-1 postchemotherapy (▲). Results are expressed as mean plus SEM of absolute neutrophil counts of groups of four mice per point.

primed mice for an enhanced neutrophil response to IL-1 administered post chemotherapy. The timing and duration of administration of IL-1 post chemotherapy was also critical with administration beginning 12 hours post 5-FU being significantly more effective than administration at the time of treatment or 2 or 24 hours post 5-FU. The duration of IL-1 treatment was found to be optimal in mice over 4 days and up to 7 days in 5-FU–treated primates. In the latter model, 5-FU (150 mg/kg) resulted in severe neutropenia for up to 30 days. Daily IL-1 (1 μg/kg/d) for 2 or 7 days reduced the duration of neutropenia to 17 days.[88] More prolonged administration of IL-1 in mice and primates was associated with increasing toxicity and could be counterproductive actually delaying recovery of neutrophils and CFU-GM.[88] Prolonged IL-1 administration induced a serum inhibitor of granulocyte colony formation and the serum suppressor activity was partially neutralized by antibodies to tumor necrosis factor α (TNFα).[88]

The ability of IL-1, alone or in combination with G-CSF, to permit dose and schedule intensification of chemotherapy was tested in mice receiving multiple cycles of CY. Chemotherapy with a weekly dose of 200 mg/kg CY for up to 12 cycles of treatment was well tolerated when combined with G-CSF or IL-1, with all cytokine-treated animals surviving and all control animals dying. We then intensified the CY dose to 300, 350, and 400 mg/kg at weekly intervals, with or without IL-1 plus G-CSF treatment. All control animals died after 1 cycle of 400 mg/kg CY, all after 2 cycles of 350 mg/kg CY, and all after 3 cycles of 300 mg/kg CY. In contrast, all cytokine-treated mice survived 3 cycles of 300 or 350 mg/kg CY. At 400 mg/kg most animals died rapidly in the first cycle regardless of whether they received cytokine treatment. This mortality was unrelated to myelotoxicity and has been attributed to an acute CY cardiotoxicity. Therefore, cytokines can permit dose escalation within the framework of myelotoxicity but it must be recognized that dose escalation will ultimately be limited by toxicities in other organ systems.

Schedule intensification can also be achieved with IL-1 plus G-CSF. Normally, rescheduling of CY treatment at weekly intervals is required, because with shorter intervals, CY would be administered at the time of neutrophil nadir at 4 to 5 days post the previous CY treatment. With IL-1 and G-CSF treatment the intervals of CY treatment can be reduced to 4 days with rapid recovery of neutrophils after each dose of CY. In contrast, noncytokine treated mice receiving CY schedule intensification had prolonged neutropenia and high mortality.

We tested the significance of dose intensification in treatment of cancer using a murine spontaneous breast tumor model.[89] Escalating doses of 5-FU (100 to 200 mg/kg) were administered each week for 3 weeks to tumor-bearing mice and groups of 10 mice were given IL-1, G-CSF, or GM-CSF alone or combinations of IL-1 with CSFs. By the third week of treatment, G- or GM-CSF–treated mice were as neutropenic as controls but IL-1–treated groups, with or without G-CSF or GM-CSF, showed rapid recovery of neutrophils. In all groups the tumor growth rate was profoundly suppressed in a chemotherapy dose-dependent manner (IL-1 or CSFs had no effect on tumor growth in the absence of chemotherapy). With chemotherapy alone effective tumor regression was associated with high mortality, with 80% of mice dying within 3 weeks, and the survivors losing 25% of their body weight. In contrast, IL-1–treated mice had only a 5% mortality and a 3% loss of body weight.[89]

IL-1 has been shown to enhance survival of otherwise lethally irradiated mice transplanted with allogeneic bone marrow.[90] Animals exhibited accelerated neutrophil recovery and donor cells developed tolerance to host major histocompatibility (MHC) antigens. Combinations of cytokines have also been used after allogeneic murine bone marrow transplantation.[91] The most effective combination for increasing the circulating neutrophil count above control values at day 7 posttransplant was the combination of IL-1 and G-CSF, followed by G-CSF alone, the combination of IL-1 plus GM-CSF, withG-CSF plus GM-CSF being least effective. In syngeneic murine bone marrow transplan-

tation we have observed that 24-hour in vitro pretreatment of bone marrow obtained from donors treated with 5-FU 24 hours previously enhanced bone marrow reconstitution and accelerated recovery of peripheral neutrophil counts in animals subsequently treated with IL-1 and G-CSF in the posttransplant period.[56]

CLINICAL STUDIES OF IL-1

We have studied escalating doses of recombinant IL-1β alone and following a myelosuppressive dose of 5-FU in patients with neutropenia.[92] A 30-minute intravenous infusion of IL-1 over a dose range of 0.002 to 0.1 μg/kg per day was given on 2 consecutive days. Transient leukopenia was followed by a 1.3- to 6.0-fold dose-dependent neutrophil leukocytosis on the days of IL-1 administration. Analysis of cytokine receptor modulation on patient neutrophils showed that G-CSF binding was downmodulated within 30 minutes to 24 hours post IL-1 infusion and then gradually recovered after 24 hours.[93] This result correlated with a rapid increase in serum G-CSF induced by IL-1. There was a less dramatic downmodulation of GM-CSF and TNF binding in the same time period, perhaps indicative of IL-1's capacity to induce in vivo production of these cytokines, albeit to a lesser extent than G-CSF. In contrast, IL-I binding was downregulated early after IL-1 infusion and then increased up to sixfold after 6 to 8 hours. This result also correlated with an increase in neutrophil superoxide generation. These receptor upmodulation and downmodulation phenomena are an in vivo phenomenon because in vitro incubation of normal neutrophils with IL-1 at 37°C for 30 minutes or 8 hours downmodulated IL-I binding in a dose-dependent manner, and did not significantly affect the receptor binding of other cytokines.[93] We have discovered two mechanisms to account for the ability of IL-1 in vivo to upregulate IL-1 receptors on myeloid cells. The first involves a glucocorticoid pathway. At 4°C, glucocorticoids did not affect cytokine binding to neutrophils, but at 37°C they produced a twofold to fivefold increase of ^{125}I-IL-I binding at 37°C on neutrophils, bone marrow cells, macrophages, and macrophage cell lines[94] (Fig 7). This induction could be suppressed by ketoconazole, a glucocorticoid antagonist. The upregulation of IL-I receptors was dose- and time-dependent process and was due to an increase in receptor number rather than affinity. Because, in vivo, IL-1 induces elevated glucocorticoid production and patients all exhibited elevated serum cortisol levels after IL-I administration,[92] this shows a novel mechanism by which IL-1 can amplify its action in vivo by increasing the expression of its own receptors on IL-1–responsive cells. A second mechanism is also involved because incubation of myeloid cells, including macrophages that express high-affinity G-CSF receptors,[95] with G-CSF in vitro also leads to rapid upregulation of the IL-I receptor (Fig 7). Furthermore, G-CSF and glucocorticoids, both elevated in serum following IL-1 treatment, synergize in increasing the expression of IL-1 R (Fig 7).

In our clinical study, an increase in platelet counts was observed a median of 14 days after IL-1β administration. In another study, patients with metastatic malignant disorders receiving five daily intravenous infusions of IL-1β at 0.001

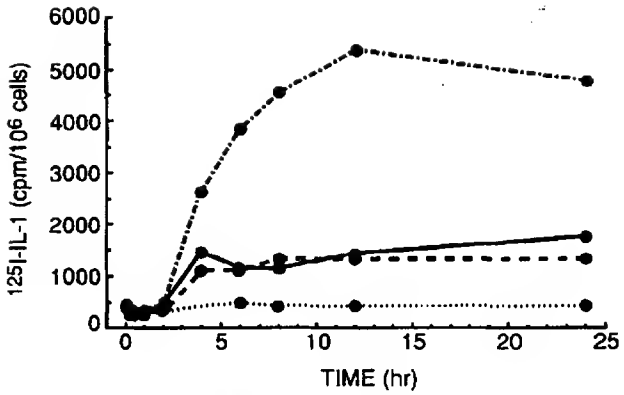


Fig 7. Synergistic interaction between rhG-CSF and corticosteroid (dexamethasone [Dex]) in the upregulation of IL-1 receptors on murine bone marrow cells. Murine femoral marrow cells, 1 × 10^6, were incubated in the presence or absence of 10 ng of G-CSF, 10^{-6} mol/L Dex, or a combination, at 37°C. Binding of ^{125}rhIL-1β was determined at various intervals over 24 hours and corrected for nonspecific binding.

and 0.01 μg/kg/d also showed a 50% increase in platelets.[96] The increase in platelet counts was first noted 6 days after treatment and was sustained for 24 days. It is probable that the platelet response is indirect via IL-1 induction of thrombopoietic cytokines such as IL-6 or IL-11, nevertheless IL-1 may be beneficial for the treatment of thrombocytopenia. While fewer days of neutropenia were observed following 5-FU plus IL-1 than after 5-FU alone, no statistically significant myeloprotection was observed. To obtain hematologic responses comparable with those observed in animal experiments, more prolonged IL-1 treatment is probably necessary, as is a requirement for combinations of IL-1 with G-CSF, GM-CSF, or IL-3. Side effects of IL-I included fever, chills, and headache in the majority of patients, and hypotension was observed at the highest dose level (0.1 μg/kg) and was dose limiting.[92,96]

NEGATIVE REGULATORS OF STEM CELL PROLIFERATION

In steady-state hematopoiesis most stem cells are in a nonproliferating state. This may be seen as a passive process involving absence of positive signals or an active process involving suppressive negative regulators. Interactions between certain positive and negative cytokines active at the stem cell level are shown in Fig 8. Negative regulators could work by blocking, downmodulating, or otherwise reducing the function of receptors for positive regulators on stem cells. They may interfere with signal transduction pathways, transcriptional factors, and mRNA production or stability, involving genes activated by positive regulators. They may also act indirectly to block synthesis of positive regulators. While a substantial body of data attests to the existence of suppressor/inhibitor factors in hematopoiesis,[97] few have, until recently, been legitimized by having their gene cloned, sequenced, and expressed.

Transforming growth factor β (TGFβ) has a substantial claim to be a physiologic negative regulator of early hematopoietic cells. TGFβI is a highly stable peptide that

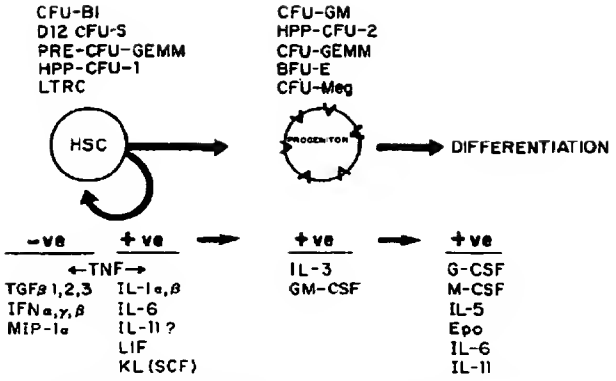


Fig 8. Cytokines provide competing signals at the primitive stem cell level (HSC). The negative signals include members of the TGFβ family and MIP-1α (specific for early cells). Interferons (IFNα, IFNβ, IFNγ) are also suppressive but are less selective, inhibiting later progenitor cell proliferation. Nevertheless, potent synergism between IFN and TNF or TGFβ exists. TNF may mediate inhibition or synergize in stimulating early cells depending on the cytokine combinations. IL-1, IL-6, and KL unequivocally act as positive stimuli for stimulation of early stem cells when used in combination with each other or with the cytokines normally active on later progenitor cells. Conflicting reports exist as to the action of leukemic inhibitory factor on HSC, and IL-11 is still under evaluation.

consists of two identical chains, each containing 112 amino acids. A number of additional closely related TGFβs have subsequently been identified and their separate genes cloned.[98] The TGFβs are highly pleiotropic, stimulating proliferation in some cells, while being a potent inhibitor in others. Moreover, they can regulate the synthesis of collagen and other critical components of the extracellular matrix with which hematopoietic cells and cytokines can interact. TGFβ is the most potent known endogenous suppressant of lymphocyte proliferation and function, and its endogenous production by both T- and B-cells acts as an autocrine-negative signal by inhibiting the action of positive signals provided by various ILs and other cytokines.[98] TGFβ inhibits murine and human BFU-E[99-101] and may[99] or may not[101] inhibit CFU-E. Megakaryopoiesis and platelet production is also inhibited, probably by inhibition of megakaryocyte endomitosis.[102] Ath action of TGFβ on myelopoiesis is more complex, and day 7 human CFU-GM have been reported to be potentiated by TGFβ,[99,100] whereas day 14 human CFU-GM are inhibited[103] as are early murine bipotent and multipotent CFU.[101] We have shown that HPP-CFC responsive to IL-1 plus CSFs are profoundly inhibited by TGFβ1 and β3 (Fig 9), whereas comparable concentrations of TGFβ either did not inhibit more committed progenitor cell (CFU-GM) cloning with G-CSF, M-CSF, or IL-3, or actually enhanced GM-CSF–induced colony formation (Fig 9). Further evidence for a selective inhibition of early stem cells was obtained in studies in which TGFβ addition to long-term bone marrow cultures blocked the proliferation of primitive hematopoietic cells that would normally be actively proliferating following a media change or IL-1 addition.[103] In vivo TGFβ1, administered locoregionally into the femoral artery, inhibited baseline and IL-3–driven proliferation of bone marrow cells and was relatively selective for the earlier multipotent progenitor cells.[104] We have shown that in vivo administration of TGFβ1 and TGFβ3 inhibited HPP-CFC entry into cycle after 5-FU–induced myelosuppression in mice. Furthermore, actively cycling HPP-CFC in regenerating marrow could be placed in a noncycling state following in vivo TGFβ administration over a period of 2 to 3 days.

The mechanism of TGFβ inhibition of stem cell proliferation is obscure but may involve a suppressor gene product such as that of the retinoblastoma gene.[105] TGFβ is also a potent inhibitor of IL-1 receptor expression on hematopoietic cells and may thus inhibit stem cells by depriving them of the ability to respond to positive cytokine signals.[106]

Analysis of stem cell proliferation using the CFU-S assay indicated that inhibitor and stimulator-producing cells existed within the bone marrow microenvironment, and their spatial distribution and proportion determined the cycle status of CFU-S.[107] Inhibitors of murine CFU-S proliferation were also identified in normal human bone marrow cultures, and production appeared to correlate with the cyclic waves of early hematopoietic cell proliferation associated with weekly media replenishment of cultures.[108] The CFU-S specific proliferation regulator (stem cell inhibitor [SCI]) also inhibited HPP-CFC in vitro, and using the latter assay it was possible to purify SCI to homogeneity and obtain sequence information.[109] These data indicated that SCI was identical to a previously described cytokine, macrophage inflammatory protein 1α (MIP-1α). MIP-1α is a peptide of 69 amino acids with a molecular mass of 7,830 daltons and is 60% identical at the amino acid level to a second member of the MIP-1 family, MIP-1β. MIP-1α and β are members of a large family of putative cytokines that are produced by activated macrophages, T cells, or fibroblasts. The biologic activities of this family are still being characterized, but so far include

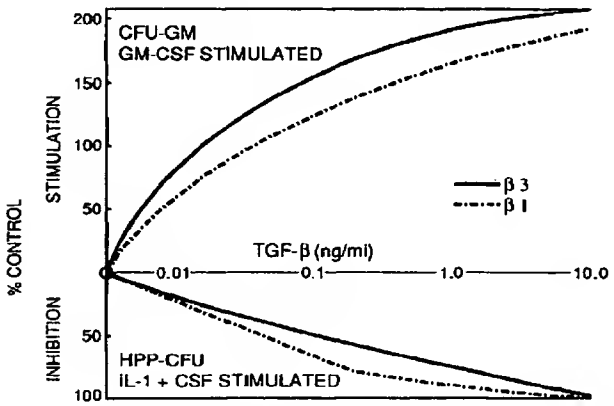


Fig 9. Inhibitory action of rhTGFβ1 and β3 on early hematopoietic precursors (HPP-CFU) and TFGβ potentiating activity on later progenitors [CFU-GM]. TGFβ was titrated in IL-1 plus GM-CSF stimulated cultures of day 4 post 5-FU bone marrow and percent inhibition of HPP determined (HPP-CFU assayed as in Fig 3). Normal bone marrow CFU-GM were assayed in 1-mL agarose cultures of 2.5 × 10^4 untreated marrow cells stimulated with 1,000 U rmGM-CSF with titrated doses of TGFβ. Stimulation is the percentage increase in day 7 colonies with TGFβ addition over the numbers stimulated by GM-CSF alone.

effects on neutrophils, monocytes, and hematopoietic cells.[110] The biologic activities of SCI/MIP-1α suggest that it may be a primary negative regulator of stem cell proliferation (Fig 8) and as such may have a therapeutic application in the protection of hematopoietic stem cells from damage during cytotoxic therapy of cancer. TNFα and lymphotoxin (LT or TNFβ) alone or in combination with interferons have been shown to inhibit in vitro hematopoiesis.[111,112] Inhibition of granulocytic, monocytic, erythroid, and multipotent progenitors has been reported, but considerable variability in results is seen that may be explained in part by the nature of the stimulus used in the various assays.[112] We have shown that in clonogenic assay of normal human marrow or using purified progenitors, cultures stimulated with G-CSF were profoundly inhibited by TNFα, with 80% inhibition of CFU-GM seen as 100 U of TNF. In contrast, in colonies stimulated by GM-CSF, 50% inhibition was seen only with greater than 1,000 U TNF, and even with 10,000 U 22% of colonies were resistant to inhibition. Thus, at the progenitor cell level, TNF inhibition was determined by the CSF species used (Fig 10). In short-term liquid culture of CD34-positive highly purified human progenitor cells, TNFα strongly potentiated IL-3– and GM-CSF–induced proliferation over 5 to 8 days.[113] This potentiating effect was also seen in 7-day clonogenic assay. However, TNFα inhibited the relatively weak growth-promoting effect of G-CSF. We compared the effect of TNF on HPP-CFC stimulated by IL-1 in combination with GM-CSF, IL-3, or G-CSF. As can be seen in Fig 10, TNF potentiated the numbers of HPP-CFC developing with IL-1 plus either IL-3 or GM-CSF, but strongly inhibited HPP-CFC developing in response to IL-1 plus G-CSF.

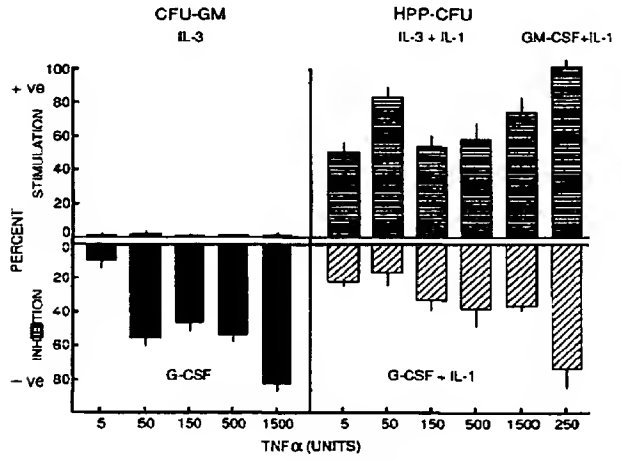


Fig 10. Potentiating and inhibiting action of TNFα in murine CFU-GM and HPP-CFU assays. CFU-GM were scored at 7 days in triplicate cultures of 5 × 10⁴ normal murine bone marrow in 1 mL of agarose medium stimulated by 1,000 U rhG-CSF or rmIL-3. Changes in cloning efficiency on addition of rmTNFα were expressed as a percentage change from controls with no TNF addition. HPP-CFU were assayed as in Fig 3, with either 1,000 U of rmGM-CSF, rmIL-3, or rhG-CSF plus 100 U rhIL-1β. Changes in HPP-CFC on addition of rmTNFα were expressed as a percentage change from controls with no TNF addition. No HPP-CFC were stimulated by TNF alone.

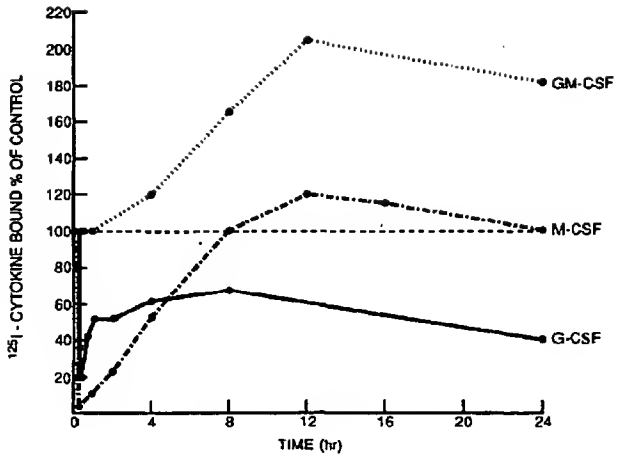


Fig 11. The influence of rmTNFα treatment on the 37°C binding of ^{125}I rmGM-CSF, rhG-CSF, and rhM-CSF to murine peritoneal macrophages. C3H/HeJ peritoneal macrophages were harvested 2 days after i.p. injection of 0.5 mL of thioglycollate medium. Cells were seeded at 4 × 10⁶ cells/mL and incubated at 37°C for 1 hour followed by washing to remove nonadherent cells. Cells were treated with rmTNF (10 ng/mL) and specific binding of the cytokines determined at various intervals after initiation of TNF treatment. Results are expressed as a percentage of binding to control non-TNF–treated macrophages cultured for comparable periods.

A possible explanation for the opposing actions of TNF on stem cells and later progenitors depending on the CSF stimulus is provided by studies on TNF-mediated receptor transmodulation on hematopoietic cells. At 37°C, TNF produced a rapid downmodulation of M-CSF receptors (cfms) on macrophages, and this was followed by recovery of receptors to normal levels within 6 to 8 hours followed by an overshoot, even in the continual presence of TNF[114,115] (Fig 11). G-CSF receptors were also downmodulated rapidly in the presence of TNF, and receptor recovery was substantially slower than in the case of M-CSF receptors[95,114] (Fig 11). In contrast, GM-CSF receptors were not downmodulated by TNF and indeed increased in number with time after TNF exposure (Fig 11). Thus, a single cytokine (TNF) acting on a specific cell type expressing multiple hematopoietic growth factor receptors can render the cell hyporesponsive to one stimulus (G-CSF), hyperresponsive to another (GM-CSF, and probably IL-3), and variably responsive, depending on time after exposure to yet another factor (M-CSF).

ANALYSIS OF THE PATHOGENESIS OF CONGENITAL CYTOPENIAS

Congenital cytopenias involving aplasia or dysplasias without obvious neoplastic transformation comprise a small but critically important group of pediatric diseases. The importance is both clinical, in that effective therapeutic strategies must be developed for these frequently life-threatening disorders, and basic, in that these "experiments of nature" give insight into critical control steps in hematopoiesis. A priori, certain broad areas of congenital deficiency can be recognized: (1) Intrinsic stem cells or

lineage-restricted progenitor cell proliferation or differentiation defects; (2) quantitative defects in hematopoietic growth factors; (3) qualitative or quantitative defects in growth factor receptors and/or signal transduction pathways; (4) aberrant activity of negative regulatory networks.

If, as is believed, the c-*kit* receptor-KL interaction is fundamental to normal stem cell control, one might expect that some human congenital cytopenias may involve mutations in human genes homologous to the murine Sl and W loci, particularly because close sequence homology between murine and human c-*kit* and KL indicates conservation. KL defects would manifest as congenital anemias/aplasias in which marrow transplantation would be ineffective but in vitro proliferative responses of stem cells and progenitor cells to KL would be normal. In contrast, c-*kit* defects would be unresponsive in vitro to KL but marrow transplantation would be effective. Table 2 is a list of syndromes associated with congenital cytopenias.

Blackfan-Diamond anemia. Blackfan-Diamond anemia and congenital aplastic anemia have been suggested as human equivalents of the Sl or W mutation. However, congenital aplastic anemia is generally responsive to allogeneic marrow transplantation, thus excluding an Sl genotype. In Blackfan-Diamond anemia there is a congenital anomaly of erythropoiesis characterized by a normochromic anemia, absent reticulocyte response, and a normocellular marrow showing markedly decreased erythroid cells with normal myeloid and megakaryocytic maturation. Approximately 70% of these patients respond to prednisone and most patients exhibit a defect in BFU-E and CFU-E. Only a small number of patients retain a relatively normal number of BFU-E and CFU-E, and even in these there is often arrest of maturation at the proerythroblast level.[116] In patients with very low (<5% of control) CFU-E and BFU-E numbers, no erythropoietin dose response can be elicited. IL-3 in vitro has been reported to increase the number and size of BFU-E, occasionally to near-normal levels.[116,117] We have studied five of these patients that represent a spectrum of in vitro response, with one extreme being a complete failure of erythropoietin (Epo) response and no BFU-E with IL-3 or GM-CSF plus Epo. BFU-E developed maximally with IL-3 plus KL plus Epo. Interestingly, this patient responded in vivo to IL-3 treatment by recovery of red blood cell parameters. In all patients studied, in vitro erythropoiesis was always maximally stimulated by KL plus Epo or KL plus IL-3 plus Epo. Clearly this responsiveness to KL excludes Blackfan-Diamond anemia as a human counterpart of the W anemic mouse where KL fails to enhance in vitro colony formation.[72] Furthermore, reports of correction of this anemia by allogeneic marrow transplantation[118] exclude a defect in the hematopoietic environment such as would be expected with a mutation/deletion at the human equivalent of the Sl locus.

Table 2. Hematopoietic Lineages and Maturation Stages Affected in Various Congenital Cytopenias

Syndrome	Level of Lesion
Kostmann	CFU-GM–CFU-G–neutrophil
Cyclic neutropenia	S.C.–CFU-GEMM–CFU-GM–neutrophil
Myelocathexis	Neutrophil
Shwechman-Diemond	S.C.–CFU-GEMM–BFU-E–neutrophil (RBC, platelet)
Dyskeratosis congenita	S.C.–CFU-GEMM–BFU-E–CFU-GM–all lineages
SCID-neutropenia	S.C.–CFU-GM–neutrophil (B cell)
Thrombocytopenia absent radii	CFU-GEMM–CFU-Meg–Meg-platelet
Amegakaryocytic thrombocytopenia	S.C.–CFU-GEMM–CFU-Meg–Meg-platelet
Fanconi's anemia	S.C.–CFU-GEMM–BFU-E–CFU-GM–all lineages
Blackfan-Diamond anemia	CFU-GEMM–BFU-E–CFU-E–RBC
Osteopetrosis	CFU-GM–CFU-M–osteoclast
Congenital aplastic anemia	S.C.–all lineages

Abbreviations: RBC, red blood cell; S.C., stem cell; CFU-Meg, CFU-megakaryocyte; SCID, severe combined immunodeficiency disease.

Schwachman-Diamond syndrome. Schwachman-Diamond syndrome is an autosomal recessive disorder characterized by an exocrine pancreatic deficiency plus chronic neutropenia, frequently with anemia, and thrombocytopenia. Marked reductions in CFU-E and CFU-GM have been reported.[119] Our own studies showed normal CFU-GM response to G-CSF and GM-CSF, but a profound suppression of myeloid colonies developing in response to IL-3. CFU-E, BFU-E, and CFU-GEMM developing in response to IL-3 plus Epo were also markedly suppressed. This finding suggests that the defect is in an early stage of multilineage hematopoietic development, particularly because IL-3 is thought to act at an earlier stage than G-CSF or GM-CSF. Nevertheless, these patients do respond in vivo to G-CSF by correction of the neutropenia.

Cyclic neutropenia. Cyclic neutropenia is an inherited disease of humans and gray collie dogs, characterized by regular (21 or 14 days, respectively) fluctuations in the number of peripheral blood cells and of bone marrow progenitor cells.[120-124] Cycling of serum or urine CSF levels has been reported,[120,122] although cure of the cyclic disorder by bone marrow transplantation suggests that the disorder is a disease intrinsic to the stem cell/progenitor cell compartment. In cyclic dogs, administration of recombinant human G-CSF, but not GM-CSF, eliminated neutrophil nadirs and marrow progenitor cell cycling[121,124] until neutralizing antibodies to the human G-CSF developed. Using recombinant dog G-CSF extended treatment totally prevented recurrent neutropenia with doses of 10 μg/kg, but with lower doses, comparable with those used in initial human trials, cycling persisted but the severity of the neutropenic episodes was markedly reduced.[124] IL-3 and GM-CSF treatment did not alter the underlying pathophysiology.

In human cyclic neutropenia, GM-CSF was ineffective in abrogating neutropenia, but G-CSF (3 to 10 μg/kg/d) shortened, but with one exception did not abrogate, neutrophil cycles.[123] Cycles were shortened from 21 days to 14 days, and the amplitude of neutrophil cycles increased but the decrease to zero neutrophils during nadirs was overcome. G-CSF also diminished the frequency and severity of infections. A cyclic pattern of progenitor cell (CFU-GM,

BFU-E) release into the circulation marks the cyclic disease, and the amplitude of this variation was increased from twofold to threefold to 10- to 100-fold on G-CSF treatment.[125] Abnormalities in progenitor cell responsiveness have been reported in cyclic neutropenia, both before and after the onset of G-CSF therapy.[125] CFU-GM were 5- to 10-fold less responsive to GM-CSF and G-CSF (but not IL-3) stimulation in vitro. BFU-E grew more readily with Epo alone than seen with normal marrow, and GM- and G-CSF–stimulated optimal numbers of myeloid colonies without further augmentation with IL-3.[125] To what extent these differences in progenitor cell responsiveness reflect differences in hematopoietic growth factor receptor numbers or affinity or receptor signal transduction defects, remains to be determined.

Kostmann-type congenital neutropenia. This is an autosomal recessive condition marked by profound neutropenia (absolute neutrophil count [ANC] $<200/mm^3$) with monocytosis and eosinophilia with neutrophil maturation arrest in marrow at the promyelocyte/myelocyte level. A number of marrow culture studies have been reported with considerable variability in results. In general, CFU-GM frequency in marrow is reported in the normal to elevated range,[126-129] although low cloning has been reported in some cases. In early studies, failure of normal neutrophil granulocyte formation in the course of colony development was reported, with monocyte/eosinophil differentiation predominating, or clear examples of terminal neutrophil differentiation but frequently with defective synthesis of primary or secondary neutrophil granules.[126,127] In retrospect, these studies were hampered by the use of stimulating factor sources that were ill-defined with respect to CSF species, eg, content of G-CSF. Furthermore, the contribution of factors in serum supplements or produced by accessory cells contributed to the variable results. With the advent of analysis with recombinant factors, it became clear that G-CSF could induce neutrophil differentiation of myeloid colonies developing in marrow cultures from the majority of Kostmann patients,[129] whereas with GM-CSF or IL-3, eosinophil/monocyte colony differentiation predominated. This failure of GM-CSF to override the neutrophil granulocyte maturation arrest in vitro (and in four of five patients treated with GM-CSF in vivo)[130] need not imply any defect in the GM-CSF induction pathway, particularly because it is increasingly apparent that optimal GM-CSF– or IL-3–induced neutrophil differentiation requires synergy with G-CSF either present in the serum supplement, or produced by accessory cells in the culture (monocytes, macrophages, fibroblasts, endothelial cells). Given an underlying heterogeneity in the syndrome, studies to date indicate a specific deficiency in the G-CSF response pathway. Analysis of G-CSF production in these patients by bioassay and immunoassay[128,129,131] has not shown a quantitative or qualitative defect in the patients ability to produce G-CSF, suggesting a defect at the receptor or receptor-signal transduction level (Table 3). This defect can clearly be overridden by the in vivo administration of pharmacologic doses of G-CSF with patient to patient variation evident by differences in the doses of G-CSF required to achieve an ANC of $\geq 1{,}000/mm^3$.[129,130]

Table 3. Conclusions in Kostmann Syndrome

(1) All patients (18) showed an increase in neutrophils from $<100/mm^3$ to 1,300 to 10,000/mm^3; usually within 8 to 9 days of G-CSF treatment. Dose requirement varied.
(2) Patients maintained functionally normal granulocyte production with daily s/c G-CSF for >3 years.
(3) Pharmacologic doses of G-CSF are required and the patient response is predicted from in vitro culture of marrow with G-CSF.
(4) G-CSF production by stromal fibroblasts and monocytes is normal by molecular (Southern, Northern), immunologic (ELISA), and biologic assay
(5) Plasma G-CSF clearance is normal to delayed. No Serum inhibitors detected.
(6) G-CSF receptors are normal in number and affinity on neutrophils and monocytes.
(7) Signal transduction defect may exist with abnormal receptor downmodulation following ligand binding.

Abbreviation: ELISA, enzyme-linked immunosorbent assay; s/c, subcutaneous.

Table 3 lists certain conclusions that can be derived from current studies of Kostmann patients. While overt changes in receptor number or affinity can be excluded, the recent isolation of two cDNAs encoding high-affinity human G-CSF receptors suggests other possibilities.[132] The two receptor forms, probably generated by alternative splicing, differ in length and in the intracytoplasmic domain. The larger form may be the predominant transcript on normal granulocytes and the shorter form may serve to sequester or transport G-CSF in other tissues. The two isoforms may differ in signal transduction properties and be abnormally expressed in Kostmann syndrome.

Congenital osteopetrosis. Juvenile osteopetrosis represents a spectrum of autosomal recessive disorders characterized by deficient osteoclast mediated bone resorption that results in the formation of dense, fragile bone. The disease is marked by encroachment of bone into marrow cavities with pancytopenia and osteosclerosis with an increased number of functionally deficient osteoclasts. The phenotype of juvenile osteopetrosis is heterogeneous, suggesting the likelihood of different diseases in humans.[133] The derivation of osteoclasts from hematopoietic stem cells and their association with the phagocytic mononuclear cell lineage led to the use of successful marrow transplantation in this disorder.[118] Qualitative abnormalities in initial bacterial killing and in bone degradation by monocytes from patients with osteopetrosis have been reported, as has diminished nitro-blue tetrazolium (NBT) reduction by their neutrophils and monocytes.[133,134] A number of animal models of autosomal recessive osteopetrosis have been reported involving defective bone resorption caused by qualitative or quantitative abnormalities of bone osteoclasts. The op/op mutant mouse suffers from congenital osteopetrosis due to a severe deficiency of osteoclasts and the total number of mononuclear phagocytes is extremely low in affected mice.[135,136] In contrast to other genetic osteopetrotic disorders, op/op mice are not cured by transplants of normal

bone marrow cells and the defect has been ascribed to an abnormal hematopoietic microenvironment. Serum, tissues, and different cell and organ conditioned media from op/op mice are devoid of biologically active M-CSF and partial correction of the defect was observed after implantation of diffusion chambers containing M-CSF producing L cells.[136] The op locus and the M-CSF gene map within the same region of the mouse chromosome 3, suggesting that the mutation is within the M-CSF gene itself. Yoshida et al[135] have sequenced M-CSF cDNA from op/op fibroblasts and found a single base pair (bp) insertion in the coding region of the M-CSF gene that generates a stop codon 21 bp downstream. To date, in an analysis of a limited series of congenital osteopetrotic children, we have not observed a defect in M-CSF production. Nevertheless, the observation that M-CSF is critical to effective osteoclast development suggests a therapeutic role for this cytokine in stimulating osteoclastic bone resorption.

CONCLUSIONS

Hematopoietic stem cells (HSC) are among the most extraordinary of somatic cell populations, possessing extensive migratory potential, highly specific homing properties, very high reproductive capacity, and multilineage differentiation potential; yet they preferentially reside in specific sites for very prolonged periods in a nonproliferating state. Figure 12 is a scheme of the marrow microenvironment that illustrates some of the multiple factors to be considered in attempting any answer to the basic question of HSC control. Both positive and negative cytokines are, in general, not constitutively produced by stromal cells, but transcription and/or translation of the various genes is rapidly induced by cytokines such as IL-1 and TNF. Under steady-state conditions the stem cells are quiescent, possibly reflecting lack of positive signals or downmodulation of receptors for these signals. The entry of small numbers of

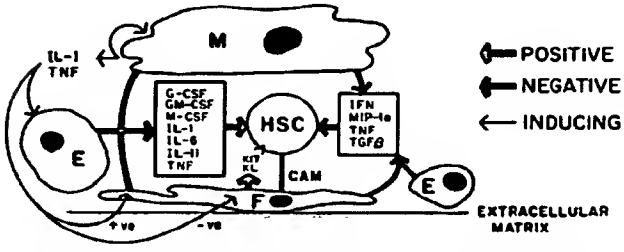


Fig 12. The interaction of the hematopoietic stem cell (HSC) with cellular and humoral influences within the bone marrow microenvironment. The attachment of the HSC via a specific cytoadhesion molecule (CAM) to the stromal "fibroblast" (F) brings its c-*kit* receptors into intimate interaction with KL expressed on stromal cells. Fibroblasts (F), endothelial cells (E), and macrophages (M) in close proximity to the HSC produce basal levels of positive growth and differentiation factors. Enhanced transcription and/or translation of multiple cytokine genes is induced by cytokine cascades involving IL-1 and TNF. Negative signals for stem cell proliferation counterbalance the positive stimuli and include TGFβ produced by stromal cells and IFNs, MIP-1α, and TNF produced by macrophages. The extracellular matrix facilitates stromal cell adhesion and can specifically bind, concentrate, and present specific cytokines.

stem cells into cell cycle with subsequent expansion and differentiation is sufficient to sustain steady-state hematopoiesis and may be the result of stochastic events influencing the movement of quiescent stem cells into cell cycle. It could equally be stochastic at the level of the stromal environment where a triggering concentration of positive signals accumulates at a local site as a consequence of low-level constitutive gene expression or binding and concentration of positive factors by their attachment to components of the extracellular matrix.

The exquisite local nature of stem cell control may be attributed to at least two known factors: a specific cytoadhesion molecule that is expressed on early stem cells and may be involved in the marrow homing properties of HSC, and the *kit*-KL interaction. The potential of the *kit*-KL system to influence cell migration is evident in the W and Sl mutations because the defects manifest during developmental stages when HSC proliferate and migrate from yolk sac to fetal liver, when germ cells also migrate from yolk sac to genital ridges, and when melanoblasts migrate from the neural crest to the skin. It has been suggested that KL expression on cell surfaces may provide highly localized special cues that could guide cell migration and homing.[74,137] In situ analysis of KL during embryogenesis has shown an association between sites of expression and cells associated with both the migrating pathway and homing sites of melanoblasts, germ cells, and hematopoietic stem cells (138).

Cloning studies of the KL gene focused on a soluble form defined by the expression of 164 to 165 amino acids of the extracellular domain.[70-72] The cell surface associated molecule has 189 amino acids in the extracellular domain, 23 hydrophobic amino acids in the transmembrane domain, and 36 amino acids in the intracellular domain.[74,137] Tissue-specific alternative splicing has been shown to generate two types of KL, both encoding a transmembrane domain but with one producing a soluble form of KL by virtue of expression of a specific proteolysis site. The other KL form has deletions of the sequences around this cleavage site and is preferentially a cell surface restricted form.[137] Within the hematopoietic microenvironment, preferential expression by specific stromal cells of the cell surface restricted form of KL would result in retention of very local control of HSC. Indeed, the *kit*-KL interaction may serve as an additional cytoadhesion mechanism for the HSC, because KL-responsive mast cells have been shown to specifically cytoadhere to Cos cells transfected with the membrane-stable form of KL.[137] In this context, the Sl^d mutant allele has a deletion of sequences encoding the transmembrane and intracellular domains of KL, and Sl^d cDNA transfected cells express in the supernatant a bioactive form of soluble KL with no detectable cell surface expression.[137] This important observation indicates that soluble KL has little ability to fulfill the normal functions of the gene product in the intact organism because Sl^d and Sl/Sl^d mice express the same pleiotropic defects as mice with a total deletion of the KL gene. These observations could imply that soluble KL is unlikely to be an effective therapeutic agent in vivo unless it is cellularly bound or associated with the extracellular

matrix. However, high doses of soluble KL in vivo can partially correct the phenotypic abnormalities (anemia, mast cell deficiency) of Sl mice.[70,72]

A further consequence of KL-kit interaction is the possibility that the cell surface form of the wild-type KL could act as a receptor and be capable of transmitting a signal to the interior of the stromal cells. Such an interaction would facilitate a highly specific signal between the HSC and its local microenvironment. The interrelationship of the HSC and its environment may be facilitated by such a signal if this in turn could lead to activation of specific cytokine genes in the KL-expressing stromal cell. A precedent for this is the potent induction of IL-7 following coculture of IL-7–dependent B cells with stromal cell lines.[139]

The role of the HSC as a target for leukemic transformation in both acute and chronic myeloid leukemia may also be seen in the context of this model of local-acting regulatory signals. Multiple pathways may lead to neoplastic transformation but there is evidence that paracrine mechanisms may be involved in which the transformed HSC constitutively produces cytokines such as IL-1 and TNF that induce stromal local cells to produce stimulatory factors. Autocrine mechanisms may lead to the development of independence of the HSC from any local-acting growth factors. More subtle mechanisms may involve loss of specific cytoadhesion, removing the transformed cell from negative regulatory influences. It is also highly likely that abnormalities in *kit*-KL interactions will be implicated in leukemogenesis.

ACKNOWLEDGMENT

I thank my colleagues, Marcus Muench, Meri Firpo, David Warren, Clay Smith, Jeff Schneider, Cristina Gasparetto, Jae-Hung Shieh, and Robert Peterson for their collaboration in these studies; Harry Satterwhite, Maureen Sullivan-James, Anne-Marie Stoppa, and Pat Sheridan for their technical assistance; and Joreatha Jones for her secretarial assistance.

REFERENCES

1. Moore MAS, Owen JJT: Stem cell migration in the developing myeloid and lymphoid system. Lancet 2:658, 1967

2. Moore MAS, Metcalf D: Ontogeny of the haemopoietic system; yolk sac origin of in vivo and in vitro colony forming cell in the developing mouse embryo. Br J Haematol 18:279, 1970

3. Kay HEM: How many cell- generations? Lancet 2:418, 1965

4. Mintz B, Anthony K, Litwin S: Monoclonal derivation of mouse myeloid and lymphoid lineages from multipotent hematopoietic stem cells experimentally engrafted in fetal hosts. Proc Natl Acad Sci USA 81:7835, 1984

5. Keller G, Snodgrass R: Life span of multipotential hematopoietic stem cells in vivo. J Exp Med 171:1407, 1990

6. Capel B, Hawley RG, Mintz B: Long- and short-lived murine hematopoietic stem cell clones individually identified with retroviral integration markers. Blood 75:2267, 1990

7. Harrison DE, Lerner C, Hoppe PC, Carlson GA, Alling D: Large numbers of primitive stem cells are active simultaneously in aggregated embryo chimeric mice. Blood 69:773, 1987

8. Micklem HS, Lennon JE, Ansell JD, Gray RA: Numbers and dispersion of repopulating hematopoietic cell clones in radiation chimeras as functions of injected cell dose. Exp Hematol 15:251, 1987

9. Van Zant G, Chen J-J, Scot-Micus K: Developmental potential of hematopoietic stem cells determined using retrovirally marked allophenic marrow. Blood 77:756, 1991

10. Jordan CT, Lemischka IR: Clonal and systemic analysis of long-term hematopoiesis in the mouse. Genes Dev 4:220, 1990

11. Till JE, McCulloch EA: A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiat Res 14:213, 1961

12. Metcalf D, Moore MAS: Haemopoietic Cells. Frontiers of Biology Series. Amsterdam, The Netherlands, North-Holland, 1971, p 71

13. Magli MC, Iscove NN, Odartchenko N: Transient nature of early haematopoietic spleen colonies. Nature 295:527, 1982

14. Visser JWM, Van Bekkum DW: Purification of pluripotent hemopoietic stem cells: Past and present. Exp Hematol 18:248, 1990

15. Spangrude GJ, Heimfeld S, Weissman LL: Purification and characterization of mouse hematopoietic stem cells. Science 241: 58, 1988

16. Szilvassy SJ, Lansdorp PM, Humphries RK, Eaves AC, Eaves CJ: Isolation in a single step of a highly enriched murine hematopoietic stem cell population with competitive long-term repopulating ability. Blood 74:930, 1989

17. Lerner C, Harrison DE: 5-Fluorouracil spares hemopoietic stem cells responsible for long-term repopulation. Exp Hematol 18:114, 1990

18. McAlister I, Wolf NS, Pietrzyk ME, Rabinovitch PS, Priestley G, Jaeger B: Transplantation of hematopoietic stem cells obtained by a combined dye method fractionation of murine bone marrow. Blood 75:1240, 1990

19. Ploemacher RE, Brons RHC: Separation of CFU-S from primitive cells responsible for reconstitution of the bone marrow hemopoietic stem cell compartment following irradiation: Evidence for a pre–CFU-S cell. Exp Hematol 17:263, 1989

20. Jones RJ, Wagner JE, Celano P, Zicha MS, Sharkis SJ: Separation of pluripotent haematopoietic stem cells from spleen colony-forming cells. Nature 347:188, 1990

21. Haskill JA, Moore MAS: Two dimensional cell separation: Comparison of embryonic and adult stem cells. Nature 26:853, 1970

22. Haskill JS, McNeill TA, Moore MAS: Density distribution analysis of in vivo and in vitro colony forming cells in bone marrow. J Cell Physiol 75:157, 1970

23. Spangrude GJ, Scollay R: A simplified method for enrichment of mouse hematopoietic stem cells. Exp Hematol 18:920, 1990

24. Spangrude GJ, Johnson GR: Resting and activated subsets of mouse multipotent hematopoietic stem cells. Proc Natl Acad Sci USA 87:7433, 1990

25. Dexter TM, Allen TD, Lajitha LG: Conditions controlling the proliferation of hemopoietic stem cells in vitro. J Cell Physiol 91:335, 1971

26. Moore MAS, Sheridan AP: Pluripotential stem cell replication in continuous human, prosimian, and murine bone marrow culture. Blood Cells 5:297, 1979

27. Moore MAS, Sheridan APC, Allen TD, Dexter TM: Prolonged hematopoiesis in a primate bone marrow culture system: Characteristics of stem cell production and the hematopoietic microenvironment. Blood 54:775, 1979

28. Donowitz GR, Quesenberry P: 5-Fluorouracil effect on cultured murine stem progeny and peripheral leukocytes. Exp Hematol 14:207, 1986

29. Spooncer E, Lord BI, Dexter TM: Defective ability to self-renew in vitro of highly purified primitive haematopoietic cells. Nature 316:62, 1985

30. van der Sluijs JP, de Jong JP, Brons NHC, Ploemacher RE: Marrow repopulating cells, but not CFU-S, establish long-term in vitro hemopoiesis on a marrow-derived stromal layer. Exp Hematol 18:893, 1990

31. Moore MAS, Broxmeyer HE, Sheridan APC, Meyers PA, Jacobsen N, Winchester RJ: Continuous human bone marrow culture: Ia antigen characterization of probable human pluripotential stem cells. Blood 55:682, 1980

32. Keating A, Powell J, Takahashi M, Singer JW: The generation of human long-term bone marrow cultures from marrow depleted of Ia (HLA-DR) positive cells. Blood 64:1159, 1984

33. Andrews RG, Takahashi M, Segal GM, Powell JS, Bernstein ID, Singer JW: The L4F3 antigen is expressed by unipotent and multipotent colony-forming cells but not by their precursors. Blood 68:1030, 1986

34. Falkenburg JHF, Fibbe WE, Goselink HM, van Rood JJ, Jansen J: Human hematopoietic progenitor cells in long-term cultures express HLA-DR antigens and lack HLA-DQ antigens. J Exp Med 162:1359, 1985

35. Bruhl P, Mergenthaler HG, Ellwart J, Dormer P: HLA-class II antigens on hemopoietic and stromal cells in human micro long-term bone marrow cultures. Exp Hematol 18:103, 1990

36. Brandt JE, Baird N, Lu L, Srour E, Hoffman R: Characterization of a human hematopoietic progenitor cell capable of forming blast cell containing colonies in vitro. J Clin Invest 82:1017, 1988

37. Caux C, Favre C, Saeland S, Duvert V, Mannoni P, Durand I, Aubry J-P, de Vries JE: Sequential loss of CD34 and class II MHC antigens on purified cord blood hematopoietic progenitors cultured with IL-3: Characterization of $CD34^-$, $HLA\text{-}DR^+$ cells. Blood 74:1287, 1989

38. Andrew RG, Singer JW, Bernstein ID: Precursors of colony forming cells in humans can be distinguished from colony forming cells by expression of the CD33 and CD34 antigens and light scatter properties. J Exp Med 169:1721, 1989

39. Brandt J, Srour EF, Besien KV, Briddell A, Hoffman R: Cytokine-dependent long-term culture of highly enriched precursors of hematopoietic progenitor cells from human bone marrow. J Clin Invest 86:932, 1990

40. Verfaillie C, Blakolmer K, McGlave P: Purified primitive human hematopoietic progenitor cells with long-term in vitro repopulating capacity adhere selectively to irradiated bone marrow stroma. J Exp Med 172:509, 1990

41. Andrews RG, Singer JW, Bernstein ID: Human hematopoietic precursors in long-term culture: Single $CD34^+$ cells that lack detectable T cell, B cell, and myeloid cell antigens produce multiple colony-forming cells when cultured with marrow stromal cells. J Exp Med 172:355, 1990

42. Lansdorp PM, Sutherland HJ, Eaves CJ: Selective expression of CD45 isoforms on functional subpopulations of $CD34^+$ hemopoietic cells from human bone marrow. J Exp Med 172:363, 1990

43. Gordon MY, Hibbin JA, Dowding C, Gordon-Smith EC, Goldman JM: Separation of human blast progenitors from granulocytic, erythroid, megakaryocytic, and mixed colony-forming cells by "panning" on cultured marrow-derived stromal layers. Exp Hematol 13:937, 1985

44. Leary AG, Ikebuchi K, Hirai Y, Wong GG, Yang Y-C, Clark SC, Ogawa M: Synergism between interleukin-6 and interleukin-3 in supporting proliferation of human hematopoietic stem cells: Comparison with interleukin-1 alpha. Blood 71:1759, 1988

45. Koike K, Ihle JN, Ogawa M: Declining sensitivity to interleukin 3 of murine multipotential hemopoietic progenitors during their development. J Clin Invest 77:894, 1986

46. Ikebuchi K, Wong GG, Clark SC, Ihle JN, Hirai Y, Ogawa M: Interleukin 6 enhancement of interleukin 3-dependent proliferation of multipotential hemopoietic progenitors. Proc Natl Acad Sci USA 84:9035, 1987

47. Ikebuchi K, Ihle JN, Hirai Y, Wong GG, Clark SC, Ogawa M: Synergistic factors for stem cell proliferation: Further studies of the target stem cells and the mechanism of stimulation by interleukin-1, interleukin-6, and granulocyte colony-stimulating factor. Blood 72:2007, 1988

48. Bradley TR, Hodgson GS, Bertoncello I: Characteristics of primitive macrophage progenitor cells with high proliferative potential: Relationship to cells with marrow repopulating ability in 5-fluorouracil treated mouse bone marrow, in Baum SJ, Ledney GD, van Bekkum DW (eds): Experimental Hematology Today. Springer International, 1982, p 285

49. Moore MAS, Warren DJ: Interleukin-1 and G-CSF synergism: *In vivo* stimulation of stem cell recovery and hematopoietic regeneration following 5-fluorouracil treatment in mice. Proc Natl Acad Sci USA 84:7134, 1987

50. Moore MAS, Warren DJ, Souza L: Synergistic interaction between interleukin-1 and CSFs in hematopoiesis, in Gale RP, Golde DW (eds): UCLS Symposium on Leukemia, Recent Advances in Leukemia and Lymphoma. New York, NY, Liss, 1987, p 445

51. McNiece IK, Kriegler AB, Quesenberry PJ: Studies on the myeloid synergistic factor from 5637: Comparison with interleukin-1 alpha. Blood 73:919, 1989

52. Welte K, Platzer E, Lu L, Gabrilove JL, Levi E, Mertelsmann R, Moore MAS: Purification and biochemical characterization of human pluripotent hematopoietic colony-stimulating factor. Proc Natl Acad Sci USA 82:1526, 1985

53. Jubinsky PT, Stanley ER: Purification of hemopoietin-1: A multilineage hematopoietic growth factor. Proc Natl Acad Sci USA 82:2764, 1985

54. Mochizuki DY, Eisenman JR, Conlon PJ, Larsen AD, Tushinski RJ: Interleukin 1 regulates hematopoietic activity, a role previously ascribed to hemopoietin 1. Proc Natl Acad Sci USA 84:5267, 1987

55. Moore MAS, Muench MO, Warren DJ, Laver J: Cytokine networks involved in regulation of haemopoietic stem cell proliferation and differentiation, in: Molecular Control of Haemopoiesis. CIBA Symposium No. 148. Chichester, Wiley-Interscience, 1990, p 43

56. Moore MAS: Role of interleukin-1 in hematopoiesis. Immunol Res 8:165, 1989

57. Warren DJ, Moore MAS: Synergism among interleukin 1, interleukin 3, and interleukin 5 in the production of eosinophils from primitive hemopoietic stem cells. J Immunol 140:94, 1988

58. Bertoncello I, Bradley TR, Hodgson GS, Dunlop JM: The resolution, enrichment, and organization of normal bone marrow high proliferative potential colony-forming cell subsets on the basis of rhodamine-123 fluorescence. Exp Hematol 19:174, 1991

59. Moreau J-F, Bonneville M, Godard A, Gascan H, Bruart V, Moore MAS, Soulillou JP: Characterization of a factor produced by human T cell clones exhibiting eosinophil-activating and burst-promoting activities. J Immunol 138:3844, 1987

60. Gascan H, Anegon I, Praloran V, Naulet J, Godard A, Soulillou J-P, Jacques Y: Constitutive production of human interleukin for DA cells/leukemia inhibitory factor by human tumor cell lines derived from various tissues. J Immunol 144:2592, 1990

61. Leary AG, Wong GG, Clark SC, Smith AG, Ogawa M: Leukemia inhibitory factor/differentiation-inhibiting activity/

human interleukin for DA cells augments proliferation of human hematopoietic stem cells. Blood 75:1960, 1990

62. Chabot B, Stephenson DA, Chapman VM, Besmer P, Bernstein A: The proto-oncogene c-kit encoding a transmembrane tyrosine kinase receptor maps to the mouse W locus. Nature 335:88, 1988

63. Bernstein SE, Russell ES, Keighley G: Two hereditary mouse anemias ($S1/S1^d$ and W/W^v) deficient in response to erythropoietin. Ann NY Acad Sci 149:475, 1968

64. Dexter TM, Moore MAS: In vitro duplication and "cure" of hematopoietic defects in genetically anemic mice. Nature 269:412, 1977

65. Nocka K, Buck J, Levi E, Besmer P: Candidate ligand for c-kit transmembrane kinase receptor: KL, a fibroblast derived growth factor stimulates mast cells and erythroid progenitors. EMBO J 9:3287, 1990

66. Huang E, Nocka K, Beyer D, Chu TY, Lam HW, Wellner D, Buck J, Besmer P: The hematopoietic growth factor KL is encoded by the Steel locus and is the ligand of the c-kit receptor, the gene product of the W locus. Cell 63:225, 1990

67. Williams DE, Eisenman J, Baird A, Rauch C, Van Ness K, March CJ, Park LS, Martin U, Mochizuki DY, Boswell HS, Burgess GS, Cosman D, Lyman SD: Identification of a ligand for the c-kit proto-oncogene. Cell 63:167, 1990

68. Anderson DM, Lyman SD, Baird A, Wignall JM, Eisenman J, Rauch C, March CJ, Boswell HS, Gimpel SD, Cosman D, Williams DE: Molecular cloning of mast cell growth factor, a hematopoietin that is active in both membrane bound and soluble forms. Cell 63:235, 1990

69. Copeland NG, Gilbert DJ, Cho BC, DonovanPJ, Jenkins NA, Cosman D, Anderson D, Lyman SD, Williams DE: Mast cell growth factor maps near the Steel locus on mouse chromosome 10 and is deleted in a number of Steel alleles. Cell 63:175, 1990

70. Zsebo KM, Wypych J, McNiece IK, Lu HS, Smith KA, Karkare SB, Sachdev RK, Yuschenkoff VN, Birkett NC, Williams LR, Satyagal VN, Tung W, Bosselman RA, Mendiaz EA, Langley KE: Identification, purification, and biological characterization of hematopoietic stem cell factor from Buffalo Rat Liver-conditioned medium. Cell 63:195, 1990

71. Martin FH, Suggs SV, Langley KE, Lu HS, Ting J, Okino KH, Morris CF, McNiece IK, Jacobsen FW, Mendiaz EA, Birkett NC, Smith KA, Johnson MJ, Parker VP, Flores JC, Patel AC, Fisher EF, Erjavec HO, Herrera CJ, Wypych J, Sachdev RK, Pope JA, Leslie I, Wen D, Lin C-H, Cupples RL, Zsebo KM: Primary structure and functional expression of rat and human stem cell factor DNAs. Cell 63:203, 1990

72. Zsebo KM, Williams DA, Geissler EN, Broudy VC, Martin FH, Atkins HL, Hsu R-Y, Birkett NC, Okino KH, Murdock DC, Jacobsen FW, Langley KE, Smith KA, Takeishi T, Cattanach BM, Galli SJ, Suggs SV: Stem cell factor is encoded at the S1 locus of the mouse and is the ligand for the c-kit tyrosine kinase receptor. Cell 63:213, 1990

73. Witte ON: Steel locus defines new multipotent growth factor. Cell 63:5, 1990

74. Flanagan JG, Leder P: The kit ligand: A cell surface molecule altered in steel mutant fibroblasts. Cell 63:185, 1990

75. McNiece IK, Langley KE, Zsebo KM: Recombinant human stem cell factor synergises with GM-CSF, G-CSF, IL-3 and Epo to stimulate human progenitor cells of the myeloid and erythroid lineages. Exp Hematol 19:226, 1991

76. Broxmeyer HE, Hangoc G, Cooper S, Anderson D, Cosman D, Lyman SD, Williams DE: Influence of murine mast cell growth factor (c-kit ligand) on colony formation by mouse marrow hematopoietic progenitor cells. Exp Hematol 19:143, 1991

77. Muench MO, Moore MAS: Interactions among colony stimulating factors, IL-1β, IL-6 and kit-ligand (KL) in the regulation of primitive murine hematopoietic cells. (submitted)

78. Moore MAS: The future of cytokine combination therapy. Cancer (in press)

79. Iscove NI, Yan XQ: Precursors (pre-CFUmulti) of multilineage hemopoietic colony forming cells quantitated in vitro. J Immunol 145:190, 1990

80. Kaizer H, Stuart R, Brookmeyer R, Beschorner W, Braine HG, Burns W, Fuller DJ, Korbling M, Mangan KF, Saral R, Sensenbrenner LL, Shadduck RK, Shende AC, Tutschka P, Yeager A, Zinkham WH, Colvin OM, Santos GW: Autologous bone marrow transplantation in acute leukemia: A phase I study of in vitro treatment of marrow with 4-Hydroxycyclophosphamide to purge tumor cells. Blood 65:1504, 1985

81. Berenson RJ, Andrews RG, Bensinger WI, Kalamasz D, Knitter G, Buckner CD, Bernstein ID: Antigen $CD34^+$ marrow cells engraft lethally irradiated baboons. J Clin Invest 81:951, 1988

82. Smith C, Gasparetto C, Collins N, Gillio A, Muench M, O'Reilly RJ, Moore MAS: Purification and partial characterization of a human pre-CFU precursor population. Blood (in press)

83. Lemoli RM, Gasparetto C, Scheinberg DA, Moore MAS, Clarkson BD, Gulati SC: Autologous bone marrow transplantation in acute myelogenous leukemia: In vitro treatment with myeloid-specific monoclonal antibodies and drugs in combination. Blood 77:1829, 1991

84. Moore MAS: Coordinate actions of hematopoietic growth factors in stimulation of bone marrow function, in Sporn MB, Roberts AB (eds): Handbook of Experimental Pharmacology–Peptide Growth Factors and Their Receptors. Berlin, Springer-Verlag, 1990, p 299

85. Benjamin WR, Tare NS, Hayes TJ, Becker JM, Anderson TD: Regulation of hemopoiesis in myelosuppressed mice by human recombinant IL-1 alpha. J Immunol 142:792, 1989

86. Castelli MP, Black PL, Schneider M, Pennington R, Abe F, Talmadge JE: Protective, restorative, and therapeutic properties of recombinant human IL-1 in rodent models. J Immunol 140:3830, 1988

87. Neta R, Vogel SN, Sipe JD, Wong GG, Nordon RP: Comparison of *in vivo* effects of human recombinant IL-1 and human recombinant IL-6 in mice. Lymphokine Res 7:403, 1988

88. Gasparetto C, Laver J, Abboud M, Gillio A, Smith C, O'Reilly RJ, Moore MAS: Effects of IL-1 on hematopoietic progenitors: Evidence of stimulatory and inhibitory activities in a primate model. Blood 74:547, 1989

89. Moore MAS, Stolfi RL, Martin DS: Hematologic effects of Il-1 beta, G-CSF and GM-CSF in tumor bearing mice treated with 5-fluorouracil. J Natl Cancer Inst 82:1031, 1990

90. Oppenheim JJ, Neta R, Tiberghien P, Gress R, Kenny JJ, Longo DL: Interleukin-1 enhances survival of lethally irradiated mice treated with allogenic bone marrow cells. Blood 74:2257, 1989

91. Atkinson K, Matias C, Guiffre A, Seymour R, Cooley M, Biggs J, Munro V, Gillis S: In vivo administration of granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage CSF, interleukin-1 (IL-1), and IL-4, alone and in combination, after allogeneic murine hematopoietic stem cell transplantation. Blood 77:1376, 199t

92. Crown J, Kemeny N, Jakubowski A, Gordon M, Sheridan C, Toner G, Meisenberg B, Gasparetto C, Wong G, Botet J, Applewhite J, Sinha S, Moore MAS, Kelsen D, Buhles W, Gabrilove J: Phase I-II trial of recombinant human interleukin-1β with and without 5-fluorouracil in patients with gastrointestinal cancer. (submitted)

93. Shieh JH, Gordon MS, Jakubowski AA, Gabrilove JL, Moore MAS: Modulation of cytokine receptors and superoxide

production in neutrophil treated with IL-1 in vitro and in vivo. Blood 76:165, 1990 (abstr, suppl)

94. Shieh JH, Peterson RHF, Moore MAS: Modulation of interleukin-1 receptors on human neutrophils by glucocorticoids. Blood 76:166, 1990 (abstr, suppl)

95. Shieh J-H, Peterson RHF, Moore MAS: Modulation of granulocyte colony-stimulating factor receptors on murine peritoneal exudate macrophages by tumor necrosis factor alpha. J Immunol 146:2648, 1991

96. Tewari A, Buhles W, Starnes HF: Preliminary report: Effects of interleukin-1 on platelet count. Lancet 336:712, 1990

97. Axelrad AA: Some hemopoietic negative regulators. Exp Hematol 18:143, 1990

98. Sporn MB, Roberts AB: Transforming growth factor-β: Multiple actions and potential clinical applications. JAMA 262: 938, 1989

99. Ottmann OG, Pelus LM: Differential proliferative effects of transforming growth factor-β on human hematopoietic progenitor cells. J Immunol 140:2661, 1988

100. Aglietta M, Stacchini A, Severino A, Sanavio F, Ferrando ML, Piacibello W: Interaction of transforming growth factor-beta 1 with hemopoietic growth factors in the regulation of human normal and leukemic myelopoiesis. Exp Hematol 17:296, 1989

101. Keller JR, McNiece IK, Sill KT, Ellingsworth LR, Quesenberry PJ, Sing GK, Ruscetti FW: Transforming growth factor β directly regulates primitive murine hematopoietic cell proliferation. Blood 75:596, 1990

102. Greenberg SM, Chandrasekhar C, Golan DE, Handin RI: Transforming growth factor β inhibits endomitosis in the Dami human megakaryocytic cell line. Blood 76:533, 1990

103. Cashman JD, Eaves AC, Raines EW, Ross R, Eaves CJ: Mechanisms that regulate the cell cycle status of very primitive hematopoietic cells in long-term human marrow cultures. 1. Stimulatory role of a variety of mesenchymal cell activators and inhibitory role of TGFβ. Blood 75:96, 1990

104. Goey H, Keller JR, Baek T, Longo DL, Ruscetti FW, Wiltrout RH: Inhibition of early murine hemopoietic progenitor cell proliferation after after in vivo locoregional administration of transforming growth factor-β1. J Immunol 143:877, 1989

105. Moses HL, Yang EY, Pietenpol JA: TGF-β stimulation and inhibition of cell proliferation: New mechanistic insights. Cell 63:245, 1990

106. Dubois CM, Ruscetti FW, Palaszynski EW, Fald LA, Oppenheim JJ, Keller JR: Transforming growth factor β is a potent inhibitor of interleukin 1 (IL-1) receptor expression: Proposed mechanism of inhibition of IL-1 action. J Exp Med 172:737, 1990

107. Lord BI, Wright EG: Spatial organization of CFU-S proliferation regulators in the mouse femur. Leuk Res 8:1073, 1984

108. Wright EG, Sheridan P, Moore MAS: An inhibitor of murine stem cell proliferation produced by normal human bone marrow. Leuk Res 4:309, 1980

109. Graham GJ, Wright EG, Hewick R, Wolpe SD, Wilkie NM, Donaldson D, Lorimore S, Pragnell IB: Identification and characterization of an inhibitor of haemopoietic stem cell proliferation. Nature 344:442, 1990

110. Wolpe SD, Cerami A: Macrophage inflammatory proteins 1 and 2: Members of a novel superfamily of cytokines. FASEB J 3:2565, 1989

111. Broxmeyer HE, Williams DE, Lu L, Cooper S, Anderson SL, Beyer GS, Hoffman R, Rubin BY: The suppressive influences of human tumor necrosis factors on bone marrow hematopoietic progenitor cells from normal donors and patients with leukemia: Synergism of tumor necrosis factor and interferon-gamma. J Immunol 136:4487, 1986

112. Moore MAS, Welte K, Gabrilove J, Souza LM: Biological activities of recombinant human granulocyte-colony stimulating factor (rhG-CSF) and tumor necrosis factor: in vivo and in vitro analysis, in Neth R, Gallo RC, Greaves MF, Kabisch H (eds): Modern Trends in Human Leukemia. Proceedings of the VII Wilsede Conference. New York, NY, Springer-Verlag, 1988, p 210

113. Caux C, Saeland S, Favre C, Duvert V, Mannoni P, Banchereau J: Tumor necrosis factor-alpha strongly potentiates interleukin-3 and granulocyte-macrophage colony-stimulating factor-induced proliferation of human $CD34^+$ hematopoietic progenitor cells. Blood 75:2292, 1990

114. Shieh J-H, Moore MAS: Hematopoietic growth factor receptors. Cytotechnology 2:269, 1989

115. Shieh JH, Peterson R, Warren DJ, Moore MAS: Modulation of colony-stimulating factor-1 receptors on macrophages by tumor necrosis factor. J Immunol 143:2534, 1989

116. Halperin DS, Freedman MH: Diamond-Blackfan Anemia: Eitology, pathophysiology, and treatment. Am J Pediatr Hematol Oncol 11:380, 1989

117. Halperin DS, Estrov Z, Freedman MH: Diamond-Blackfan Anemia: Promotion of marrow erythropoiesis in vitro by recombinant interleukin-3. Blood 73:1168, 1989

118. O'Reilly RJ, Brochstein J, Dinsmore R, Kirkpatrick D: Marrow transplantation for congenital disorders. Semin Hematol 21:188, 1984

119. Suda T, Mitzoguchi H, Miura Y, Kubota K, Ikuta K, Sasaki H, Nagao T: Hemopoietic colony-forming cells in Shwaehman's syndrome. Am J Pediatr Hematol Oncol 4:129, 1982

120. Moore MAS, Spitzer G, Metcalf D, Penington DG: Monocyte production of colony stimulating factor in familial cyclic neutropenia. Br J Haematol 27:47, 1974

121. Lothrop CD, Warren DJ, Souza LM, Jones JB, Moore MAS: Correction of canine cyclic hematopoiesis with recombinant human granulocyte colony stimulating factor. Blood 72:1324, 1988

122. Dunn CDR, Jones JB, Lange RD, Wright EG, Moore MAS: Production of presumptive humoral haematopoietic regulators in canine cyclic haematopoiesis. Cell Tissue Kinet 15:1, 1982

123. Hammond WP IV, Price TH, Souza LM, Dale DC: Treatment of cyclic neutropenia with granulocyte colony-stimulating factor. N Engl J Med 320:1306, 1989

124. Hammond WP, Boone TC, Donahue RE, Souza LM, Dale DC: A comparison of treatment of canine cyclic hematopoiesis with recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF), G-CSF, interleukin-3, and canine G-CSF. Blood 76:523, 1990

125. Migliaccio AR, Migliaccio G, Dale DC, Hammond WP: Hematopoietic progenitors in cyclic neutropenia: Effect of granulocyte colony-stimulating factor in vivo. Blood 75:1951, 1990

126. L'Esperance P, Brunning R, Good RA: Congenital neutropenia: In vitro growth of colonies mimicking the disease. Proc Natl Acad Sci USA 70:669, 1973

127. Zucker-Franklin D, L'Esperance P, Good RA: Congenital neutropenia: An intrinsic cell defect demonstrated by electron microscopy of soft agar colonies. Blood 49:425, 1977

128. Abboud M, Firpo M, Laver J, Warren D, Shieh JH, Gabrilove J, O'Reilly R, Moore MAS: Studies of G-CSF production and response in congenital neutropenia. Exp Hematol 17:671, 1989 (abstr)

129. Bonilla MA, Gillio AP, Ruggerio M, Kernan NA, Brochstein JA, Abboud MA, Fumagalli L, Vincent M, Welte K, Souza LM, O'Reilly RJ: Effects of recombinant human granulocyte colony-stimulating factor on neutropenia in patients with congenital agranulocytosis. N Engl J Med 320:1574, 1989

130. Welte K, Zeidler C, Reiter A, Muller W, Odenwald E, Souza L, Riehm H: Differential effects of granulocyte-macrophage colony stimulating factor (GM-CSF) and granulocyte colony stimulating factor (G-CSF) in children with severe congenital neutropenia. Blood 75:1056, 1990

131. Pietsch T, Buhrer C, Mempel K, Menzel T, Steffens U, Schrader C, Santos F, Zeidler C, Welte K: Blood mononuclear cells from patients with severe congenital neutropenia are capable of producing granulocyte colony-stimulating factor. Blood 77:1234, 1991

132. Larson A, Davis T, Curtis BM, Gimpel S, Sims JE, Cosman D, Park L, Sorensen E, March C, Smith CA: Expression cloning of a human G-CSF receptor: A structural mosaic of hematopoietin receptor, immunoglobulin and fibronectin domains. J Exp Med 172:1559, 1990

133. Shapiro F, Key L, Constantine A: Variable osteoclast appearance in human infantile osteopetrosis. Calcif Tissue Int 43:67, 1988

134. Reeves J, Arnaud S, Gordon S, Subrayan B, Block M, Huffer W, Arnaud C, Mundy G, Haussler M: The pathogenesis of infantile malignant osteopetrosis: Bone mineral metabolism and complications in five infants. Metab Bone Dis Rel Res 3:135, 1981

135. Yoshida H, Hayashi S-I, Kunisada T, Ogawa M, Nishikawa S, Okamura H, Sudo T, Shultz LD, Nishikawa S-I: The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. Nature 345:442, 1990

136. Wiktor-Jedrzeiczak W, Bartocci A, Ferrante W, Ahmed-Ansari A, Sell KW, Pollard JW, Stanley ER: Total absence of colony-stimulating factor 1 in the macrophage-deficient osteopetrotic (op/op) mouse. Proc Natl Acad Sci USA 87:4828, 1990

137. Flanagan JG, Chan DC, Leder P: Transmembrane form of the kit ligand growth factor is determined by alternative splicing and is missing in the $S1^d$ mutant. Cell 64:1025, 1991

138. Matsui Y, Zsebo KM, Hogan BLM: Embryonic expression of a haematopoietic growth factor encoded by the Sl locus and the ligand for c-kit. Nature 347:667, 1990

139. Sudo T, Ito M, Ogawa Y, Iizuka M, Kodama H, Kunisada T, Hayashi S-I, Ogawa M, Sakai K, Nishikawa S, Nishikawa S-I: Interleukin 7 production and function in stromal cell-dependent B cell development. J Exp Med 170:333, 1989

BLOOD

The Journal of
The American Society of Hematology

VOL 82, NO 12 DECEMBER 15, 1993

REVIEW ARTICLE

Hematopoietic Regulators: Redundancy or Subtlety?

By Donald Metcalf

HEMATOPOIESIS requires a highly complex series of cellular events in which a small population of stem cells needs to generate continuously large populations of maturing cells in eight major lineages. Normally, the diverse proliferative, differentiative, and maturation events required to achieve this occur with precision, which leads to the expectation that the regulatory mechanisms involved would need to be complex. It can also be anticipated that the entry of mature cells into the circulation, their selective localization in appropriate tissues, and their functional activation are also events requiring sophisticated regulatory control.

Given the likely complexity of these events and the knowledge that much of this regulation is achieved by the use of regulatory molecules that can be humoral or cell-associated, it is not surprising that many such regulatory molecules have been characterized, purified, and produced in recombinant form. The known regulators with proliferative effects on one or other hematopoietic population already exceed 20 in number and to these need to be added a variety of inhibitory factors and a number of factors allowing selective cell-cell adhesion. Many additional candidate factors are in the early phase of characterization.

This degree of complexity in biologic processes is familiar enough to those who have addressed the details of coagulation or complement activation. However, for some of those working with the proliferative hematopoietic regulatory factors, there are aspects of the regulators so far characterized that have a raised a growing conviction that these regulators may exhibit a high degree of redundancy. In short, there appear to be more regulators with similar or overlapping actions than would seem to be really necessary to achieve the required cell proliferation.

EVIDENCE FOR REDUNDANCY

There are several types of observation that support the contention that there could be redundancy amongst the proliferative hematopoietic regulators.

Common proliferative actions. Hematopoietic cell proliferation cannot occur spontaneously and needs stimulation by specific regulatory factors.[1] The nature of the events occurring is shown more clearly in semisolid than in liquid cultures because the semisolid matrix allows the progeny of individual precursor cells to remain physically localized during the process of colony formation.

Although most of the proliferative regulators can stimulate one or another type of colony formation in vitro, it needs to be emphasized that the precise pattern of colony formation stimulated by each purified regulator is quite distinctive. No two regulators stimulate exactly the same pattern of colony formation as judged either by colony numbers or, more importantly, by the lineage and maturation pattern of the cells making up the developing colonies. For example, each of the four colony-stimulating factors (CSFs) stimulates the formation of a quite distinct array of colonies.[2-5] However, if these obvious differences are ignored and attention is focused on one particular colony type, then it becomes both intriguing and puzzling to realize that more than one factor can stimulate the formation of what appears to be exactly the same type of colony. For example, the development of small maturing neutrophilic granulocytic colonies can be stimulated, apparently by direct action, by granulocyte-CSF (G-CSF), granulocyte-macrophage-CSF (GM-CSF), macrophage-CSF (M-CSF), Multi-CSF (interleukin-3 [IL-3]), stem cell factor (SCF), and IL-6. Similarly, at least seven factors can stimulate or potentiate the formation of megakaryocyte colonies of one type or another (Fig 1) and a similar situation exists for eosinophils and mast cells.

From the limited viewpoint of achieving in vivo the proliferation of granulocytes or megakaryocytes, the existence of

From The Walter and Eliza Hall Institute of Medical Research, Royal Melbourne Hospital, Victoria, Australia.

Submitted August 23, 1993; accepted September 25, 1993.

Supported by the Anti-Cancer Council of Victoria, the National Health and Medical Research Council, Canberra, the Cooperative Research Centre for Cellular Growth Factors and the National Institutes of Health, Bethesda, MD (CA-22556).

This review is based on a Presidential Symposium Address delivered at the Annual Meeting of The American Society of Hematology in Anaheim, CA, December 1992.

Address reprint requests to Donald Metcalf, MD, The Walter and Eliza Hall Institute of Medical Research, PO Royal Melbourne Hospital, 3050 Victoria, Australia.

0006-4971/93/8212-0030$3.00/0

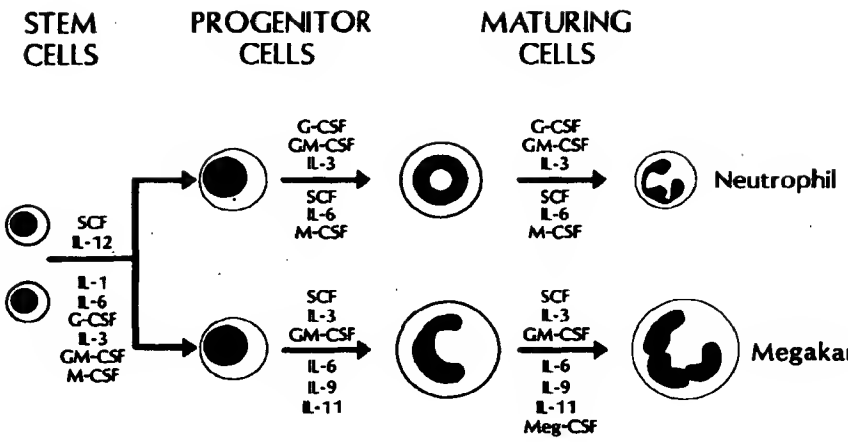


Fig 1. Multiple regulators have actions controlling the formation by stem cells of progenitor cells and, similarly, in the formation of neutrophils or megakaryocytes, six or seven regulators have been shown to have some proliferative effects.

six or seven regulators able to accomplish this process seems unnecessary. The arrangement appears not only to be redundant but to entail an inbuilt risk of dysregulation if differing signals can induce the synthesis of these different proliferative factors.

Common spectra of biologic actions. The action of individual hematopoietic regulators is usually not restricted to cells of a single lineage. Even erythropoietin, possibly the most restricted regulator, with its dominant action on mature erythroid precursors,[6] probably also has some action on megakaryocyte precursors.[7] At the other extreme, Multi-CSF (IL-3) has obvious actions on stem cells, erythroid, granulocytic, macrophage, eosinophil, megakaryocyte, mast, and probably B-lymphocyte precursors.[5,8] It is these cross-lineage actions that lead to the accumulation of multiple regulators with actions on cells in an individual lineage.

What has been more intriguing has been the growing awareness that some regulators have an astonishing range of responding target cells. The regulators concerned are leukemia inhibitory factor (LIF),[9] IL-6,[10] oncostatin-M (OSM),[11] and IL-11.[12] Although the overlap in their range of biologic actions is not complete, nevertheless two or more of these have similar actions in stimulation of platelet formation, induction of differentiation in myeloid leukemic cells, stimulation of hepatocytes to produce acute-phase protein, alteration of neuronal signalling, and suppression of lipid transport to adipocytes. Apart from a general dilemma in understanding the rationale for the existence of regulators with such a bizarre range of target cells,[13] it is equally intriguing that there should be a set of such regulators with a comparable and overlapping range of actions. These similarities extend to in vivo actions because, for example, the injection of LIF, IL-6, or IL-11 induces broadly similar increases in platelet numbers in terms of their speed of development and magnitude.[14-16]

Shared receptor subunits. Recent data derived from the cloning of cDNAs encoding the specific membrane receptors for the hematopoietic and other growth factors have shown the existence of two distinct families of related receptors: (1) tyrosine kinase receptors, including those for epidermal growth factor, M-CSF,[17] and SCF[18]; and (2) hematopoietic receptors, not containing a tyrosine kinase domain, but exhibiting obvious homology in their extracellular domains.[19] This latter group includes the receptors for erythropoietin,[20] GM-CSF,[21] Multi-CSF,[22] G-CSF,[23] IL-4,[24] IL-5,[25] IL-6,[26] IL-7,[27] LIF,[28] and IL-2.[29] The relatedness of receptors in this latter group certainly raises the likelihood that these receptors are derived by evolutionary divergence from a common ancestral receptor.

What has been even more interesting is that most of this latter group of receptors exist in high-affinity form as heterodimers. After ligand binding, the specific α-chains referred to above become associated with at least one other receptor chain (the β-chain). The intriguing aspect of this arrangement is that the β-chains involved are promiscuous. Thus the α-chains for GM-CSF, Multi-CSF, and IL-5 share the same β-chain[30] and, although the exact arrangement remains to be clarified, receptor complexes for IL-6, LIF, OSM, and IL-11 all share a common β-chain, ie, the gp130 molecule.[31-33] Because the β-chain is prominent in initiating intracellular signalling, this arrangement offers an appealing structural explanation for the observations that GM-CSF, IL-3, and IL-5 all have in common the ability to stimulate eosinophil proliferation and that LIF, IL-6, OSM, and IL-11 exhibit shared pleiotropic actions.

However, the converse aspect of this promiscuity is that the arrangement seems to document the existence of true redundancy. If one assumes for the moment that the β-chain is wholly responsible for signalling, then there would seem to be little need for the existence of three distinct α-chains on eosinophils or for three separate regulatory factors to activate such α-chains.

Coexpression of regulator receptors on individual cells. One way of explaining this apparent redundancy would be to propose that major heterogeneity exists within populations of each lineage at each differentiation stage. Perhaps only a subset of granulocytic progenitor cells expresses receptors for G-CSF, another subset of progenitors only receptors for GM-CSF, and so on. Each regulator might therefore act on its own "private" subset of responding cells. An extension of this concept might even propose that distinct subsets of mature granulocytes exist, each with

a distinctive functional capability. Differing emergency circumstances might then require the use of different regulators to stimulate the formation of the most appropriate subset of mature granulocytes to respond to the special requirements of a particular situation.

Present information is not quite complete enough to absolutely exclude this heterogeneity hypothesis. Nevertheless, in the case of the CSFs, a variety of data from autoradiographic studies using radiolabeled regulators[34-36] and the reciprocal transfer of developing colonies from one stimulus to another[3,37] suggest that the more likely situation is that a majority of cells, whether progenitor cells or maturing progeny, coexpress receptors for more than one regulator.

Indeed, the ability to document interactions between different regulators (either downregulation[38] or upregulation[39]) and a variety of competitive or enhancing interactions between pairs of regulators necessitates the coexpression of more than one type of receptor on individual cells. Any interpretation of the significance of multiple regulators with similar actions therefore needs to encompass the fact that multiple regulators can and do act on individual cells.

INTERPRETATIONS OF THE SIGNIFICANCE OF APPARENT REDUNDANCY

Consideration of these observations has led many to conclude that the hematopoietic regulators do indeed exhibit significant redundancy. Predictably, this possibility has elicited a varicty of responses. Some have mentally shrugged and concluded that, given our evolutionary origin, it is inevitable that "genetic debris" will persist in the most advanced organisms. This view would hold that some of today's "regulators" in fact perform no real function in the body, even though the regulators encoded can be shown still to have actions on present day cells. This conclusion seems illogical. If such regulators can act, then there are presumably situations in which they do act.

A less extreme response is to propose that a hierarchy of regulators exists, some with a mandatory role in daily cell production and others with weaker actions or perhaps only becoming important under emergency circumstances. A rather naive corollary of this view, implicit in many publications, is that if a factor is usually present in the circulation, it is more likely to be a major regulator than one less often or rarely detectable in the circulation.

A more appealing concept is that multiple regulators exist because they are designed to act sequentially within a particular lineage, some preferentially acting early in a differentiation sequence, others acting later in the sequence. Unfortunately this concept is not well supported by available data. Erythropoietin is perhaps the best candidatc for such a sequentially acting regulator, having its most obvious action on the most mature erythroid precursors,[6] whereas IL-3[5] and SCF[40] clearly are able to act earlier in the erythroid lineage. Similarly, IL-5 may act primarily on more mature eosinophil precursors[41] and IL-6 may act particularly on more mature megakaryocyte precursors.[42] However, these likely examples of sequential action are few in number and the bulk of the hematopoietic regulators exhibit little evidence of stage restriction. For example, each of thc four CSFs has actions on cells ranging from the stem cell to fully mature cells.[43] The notion of sequential action has done little therefore to resolve the redundancy problem.

EFFECTS OF DELETION OR SUPPRESSION OF HEMATOPOIETIC REGULATORS

One might suppose that the most definitive approach to establishing whether a particular regulator is genuinely redundant would be to suppress the regulator either using specific antibodies or by generating animals in which the gene in question has been deleted or functionally inactivated. If some abnormality develops in hematopoiesis as a result of either procedure, clearly the factor concerned is not redundant and its loss cannot be compensated for by the overlapping action of another factor.

In early studies, injection of antibodies to erythropoietin suppressed erythropoiesis,[44] indicating that the action of erythropoietin cannot be redundant. Somewhat comparable data have been generated by Hammond et al[45] from an analysis of dogs developing antibodies to injected human G-CSF that cross-neutralize canine G-CSF. Such dogs developed neutropenia, indicating that G-CSF plays an important role at least in the basal production of granulocytes, a role that is not able to be compensated for by other granulocyte-active factors such as GM-CSF or Multi-CSF.

In parallel studies, mice with a genetic defect resulting in osteopetrosis (op/op) have been recognized to have an inactivating nucleotide insertion in the M-CSF gene preventing the production of M-CSF.[46] Such mice exhibit a major deficiency in macrophage-derived osteoclasts and partial deficiencies in other macrophage populations, abnormalities correctable by the injection of M-CSF.[47] Again, the conclusion is able to be drawn that M-CSF is essential for the formation of osteoclasts and some macrophages. Because op/op mice are not completely devoid of macrophages, the formation of at least some macrophage populations must be able to be stimulated by other factors, such as GM-CSF.

At present, a number of groups are developing mice with deletion or inactivation of other hematopoietic growth factor genes such as GM-CSF or IL-3 and data from such animals may provide a much clearer view of the redundancy question.

However, some caution needs to be exercised in interpreting such data. Studies with LIF-deleted mice illustrate this comment. As mentioned above, the curious pleiotropic effects of LIF are duplicated to varying degrees by the actions of three other regulators, IL-6, OSM, and IL-11, leading to an expectation that deletion of the LIF gene might result in no observable effects. Superficially, this has indeed been what was observed, in the sense that living LIF-deleted animals can be generated.[48,49] However, such mice have been reported to be somewhat undersized and to have reduced numbers of stem and progenitor cells in the spleen and to a lesser degree in the marrow,[49] raising the possibility that some hematopoietic cells do depend for optimal performance on a special action of LIF. More importantly, LIF-deleted mice are unable to become pregnant.[48,49] In normal pregnancy, cells in the uterine wall adjacent to the implan-

tation site exhibit a marked increase in the transcription and production of LIF at the time of implantation.[50] This appears to be essential for implantation because LIF-deleted blastocysts can implant normally in LIF-producing mice and the implantation defect in LIF-deleted mice can be corrected by the injection of LIF. So, is LIF a redundant or a nonredundant regulator? The answer depends entirely on the context. Some functions of LIF may be redundant whereas at least one must be unique and crucial for pregnancy to occur.

The ability of the deletion or inactivation approach to obtain answers to the redundancy problem may in fact be a more difficult task than was envisaged by the exponents of this approach. Deletion of a growth factor gene may well induce no obvious abnormality until a particular challenge situation is applied, when a deficiency then becomes evident. Worse, it may become necessary to perform the observations at a quite restricted time after deletion. It has recently been reported that the dramatic defects in op/op mice resolve spontaneously as the animals age.[51] This suggests that, although compensating mechanisms may require some time to become operative, genuine defects may be missed if analysis is delayed too long.

My own expectation is that the present series of gene inactivation experiments may generate some initially misleading data but will ultimately result in the conclusion that most of the factors under discussion have both unique and redundant actions, depending on the circumstances analyzed.

REGULATOR COMBINATIONS ARE NECESSARY FOR OR ENHANCE HEMATOPOIETIC CELL PRODUCTION

A more positive approach to the redundancy question is to carefully analyze the consequences of using combinations of regulators compared with the effects of individual regulators, when acting alone. Are there cell populations requiring costimulation by different regulators for proliferation? Do regulator combinations induce enhanced proliferative responses? On this latter question, if equivalent concentrations of two mutually redundant factors were combined to stimulate a target progenitor cell population, one of two outcomes should be observed. The combination might exhibit the same proliferative stimulation as either acting alone or, more likely, the combination would stimulate proliferation equivalent to that stimulated by twice the concentration of either factor when acting alone.

Proliferative responses can most readily be analyzed in simplified culture systems and here the use of clonal cultures is particularly revealing because two quite different parameters can be distinguished: alteration in colony size and alteration in colony numbers. As shown in Fig 2. enhanced cell production can be based on two quite different processes and it is suggested that separate terms be adopted to distinguish between the two. Increased colony size can be labeled "synergy." This is the process by which two or more active factors, acting on the same progenitor cell, induce the formation of larger number of progeny. The second process could be termed "recruitment." In this process, the combined actions of two or more regulators allow increased numbers of progenitor cells to proliferate either because distinct subsets of progenitor cells exist that respond exclusively to one factor or because some progenitors require simultaneous stimulation by two or more factors before being able to respond.

Stem cells purified to homogeneity by fluorescence-activated cell sorter (FACS) fractionation characteristically are not able to proliferate if stimulated by single regulatory factors. Combinations of two or more regulators including SCF or IL-12 with IL-3, IL-6, and G-CSF are required before proliferation occurs.[52-54] This appears to be a clear example of recruitment (the need for simultaneous signalling by two or more regulators), but the actual cellular processes could be quite complex. Information remains incomplete on what regulator receptors are normally expressed on murine stem cells. Certainly receptors for SCF are present and, in studies in progress in this laboratory, receptors for IL-1, IL-3, IL-6, and G-CSF appear to be present in somewhat low numbers on at least a proportion of purified stem cells (C.-L. Li, G.R. Johnson, W. McKinstry, unpublished data, May 1993). SCF, when acting alone, maintains the survival of the stem cells but induces little cell division[53] and SCF may act to induce or increase expression of other receptors whose activation by the appropriate regulator is then necessary for cell division.

In contrast, the enhancement interactions noted with unfractionated or FACS-purified granulocyte-macrophage progenitor cells more often appear to represent synergy. For example, when two CSFs are combined, there is not an additive increase in resulting colony numbers, presumably because most progenitors are able to respond to stimulation by either factor when acting alone. However, what does occur is an increase in cell numbers in the colonies that is somewhat greater than achievable by using twice the concentration of either factor alone.[4,55,56]

Several mechanisms could explain synergy. Activation of additional receptors on a cell by the use of two factors is likely to enhance the mitotic signal because of coalescence of initially differing signalling pathways into a common final pathway reaching the nucleus.[57] Superadditive responses to regulator combinations could occur if signalling from one type of receptor is being restricted by limiting concentrations of some signalling intermediate. This deficiency might be overcome by supplementation of signalling intermediates from a second type of activated pathway. It has also been documented that activation of one set of receptors can enhance expression of receptors for other regulators, allowing enhanced responsiveness to the second regulator.[39] The occurrence of superadditive synergy is not the response expected from the combination of two redundant factors and is thus an argument against such redundancy.

A more dramatic enhancement of cell proliferation is observed when SCF is combined with G-CSF, GM-CSF, Multi-CSF, or IL-6 to stimulate cultures.[58-60] Again, there is not an additive increase in total colony numbers but there is a consistent 10- to 20-fold increase in mean numbers of cells per colony. Analysis of this phenomenon has shown that the key process relates to the behavior of blast colonies in the

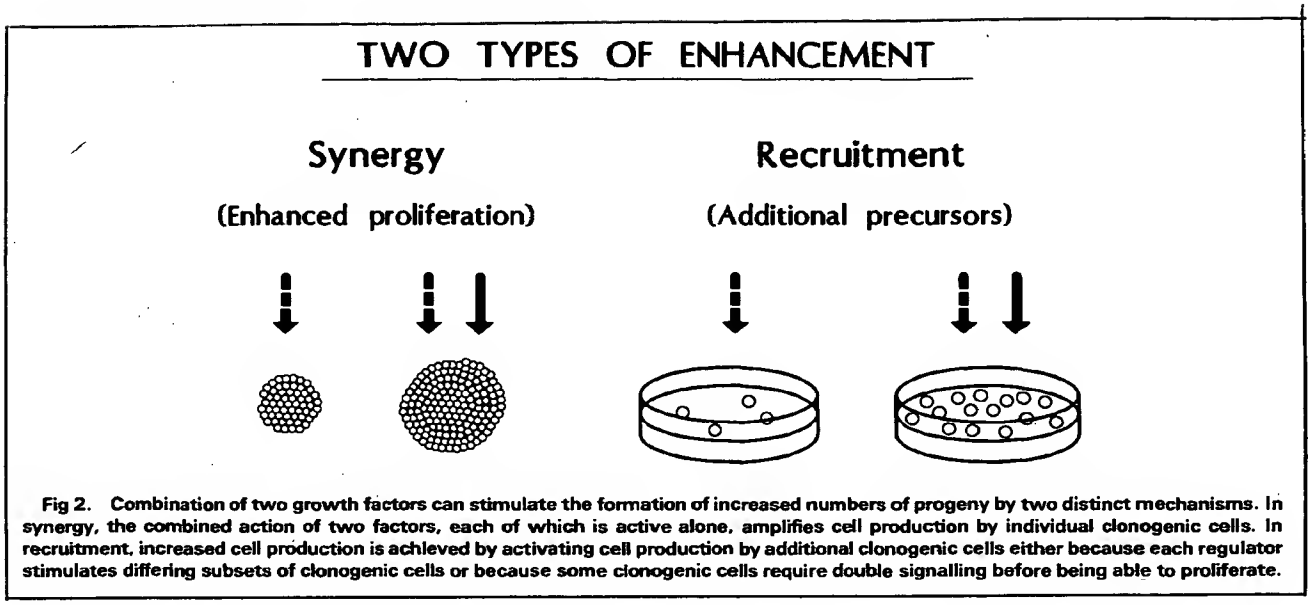


Fig 2. Combination of two growth factors can stimulate the formation of increased numbers of progeny by two distinct mechanisms. In synergy, the combined action of two factors, each of which is active alone, amplifies cell production by individual clonogenic cells. In recruitment, increased cell production is achieved by activating cell production by additional clonogenic cells either because each regulator stimulates differing subsets of clonogenic cells or because some clonogenic cells require double signalling before being able to proliferate.

cultures. These are formed (albeit rather ineffectively) by relatively mature stem cells in response to stimulation by SCF, possibly with the assistance of low levels of endogenously produced regulators of other types.[59] Deliberate addition of a CSF or IL-6 to SCF-stimulated cultures does not increase the number of these blast colonies but greatly amplifies the number of cells present in them.[60,61] The cells in blast colonies are in fact committed progenitor cells for various hematopoietic lineages, so, in this situation, enhancement is occurring by the process of synergy. However, the resulting progenitor cells are quite heterogeneous and many are in lineages not able to be stimulated to proliferate by the SCF-CSF combination used to initiate their formation.[60,61] Thus, for example, the combined action of SCF plus G-CSF leads to the production of large numbers of macrophage-committed progenitor cells. To reap the benefit of these additional progenitor cells in terms of mature cell production, the use of an additional macrophage-active factor becomes mandatory. Thus, a process that involves synergy results in a situation requiring recruitment to yield the maximum numbers of maturing progeny.

The data discussed indicate that, for optimal progenitor cell production by stem cells, at least two factors are needed and then, for optimal production of maturing progeny from these progenitor cells again, more than two factors are needed. These phenomena probably document some of the reasons why multiple regulatory factors exist. That this phenomenon is not a curious in vitro artefact has become evident from studies in vivo. The injection of G-CSF induces impressive increases in blood granulocyte levels but, curiously, also induces up to 100-fold increases in peripheral blood levels of progenitor cells in all lineages.[62] Use of G-CSF in mice with the Steel or W^v mutations that lack the ability either to produce SCF or SCF receptors is much less effective in stimulating granulopoiesis and in elevating progenitor cells,[63] allowing the conclusion that G-CSF responses are in fact SCF–G-CSF responses. The puzzling increase in "inappropriate" progenitor cells in response to G-CSF is then an example of the consequences of the combined action of SCF and G-CSF on stem cells as analyzed in vitro. The implication is that the clinical use of additional, more broadly acting regulators in such patients might achieve significant increases in monocytes, eosinophils, and erythroid cells, based on the increased numbers of progenitor cells now available for stimulation.

Not all combinations of factors lead to enhanced responses. Thus, combination of SCF with M-CSF does not result in striking enhancements.[60] Furthermore, at least one combination, that of GM-CSF with M-CSF when acting on murine cells, actually results in a significant decrease in the formation of certain macrophages compared with that able to be elicited by M-CSF acting alone.[4,64] This is not likely to be the only example of a suppressive consequence after regulator combination and this potential outcome needs to be kept in mind.

Most data indicate that stem cell proliferation requires costimulation by multiple regulatory factors, whereas progenitor cells can be stimulated effectively (if not optimally) by single regulators. However, it needs to be commented that there are some progenitor cells whose proliferation requires costimulation. For example, there is a subset of granulocyte-macrophage progenitor cells able to produce large numbers of progeny, but such proliferation requires the use of two or more CSFs.[4] Similarly, the development of maximal numbers of megakaryocyte colonies requires the combination of Multi-CSF (IL-3) with IL-6 and SCF. Thus, there appear to be situations in which it is not merely more efficient to use receptor combinations to stimulate cell formation but the use of combinations is actually mandatory. There is no reason to suppose that the same principles do not apply for the production of basal numbers of mature cells. It may always be more efficient to use multiple factor combinations to achieve any level of cell production.

A quite distinct possible advantage from the use of regula-

tor combinations may be the lower demands placed on factor-producing cells and their induction systems. It is fairly common for individual cells such as lymphocytes,[65] endothelia,[66] or stromal cells[67] to simultaneously produce more than one type of regulatory molecule in response to a single type of inductive signal. It has yet to be established whether there is a significant limitation in the ability of a cell to produce any one factor. If so, an arrangement lowering the required concentrations of each factor by using them in combination might usefully reduce demands on factor-producing tissues.

At the clinical level, the increased cell production able to be stimulated by combinations of factors compared with that achievable using single factors would have some potential advantages. Less of any one factor would need to be used, probably reducing the likelihood of adverse responses and possibly reducing costs of therapy.

REGULATOR COMBINATIONS ACHIEVE BROADER CELLULAR RESPONSES

It is now well recognized that all regulators are able to influence hematopoietic cells of more than one lineage, but the breadth of such actions differs widely. For example, at least when acting on committed progenitor cells, G-CSF action is largely restricted to granulocytic populations and M-CSF action is similarly restricted to monocyte-macrophage populations. In contrast, both GM-CSF and Multi-CSF can stimulate the production of a much broader range of hematopoietic cells. We remain quite ignorant regarding the range of cell types required for optimal responses to various emergencies such as tissue injury or infections, but for most common emergencies, it can be presumed that an optimal response does require the involvement of cells from more than one lineage. Combination of two or more CSFs in vitro increases the range of responding cells[4] and such combinations could therefore be expected to elicit broader, and thus more useful, responses in at least some clinical situations.

When various CSFs were injected into animals, the range of responding cells faithfully reproduced the range of actions established by earlier in vitro studies. However, a further subtlety emerged from these studies best exemplified by comparing the responses to G-CSF and GM-CSF when injected into the peritoneal cavity. G-CSF elicited little increase in peritoneal cell numbers but major increases in peripheral blood granulocytes.[43] In contrast, with the doses used, GM-CSF induced only minor increases in blood cell levels but a remarkable increase in peritoneal cell macrophages and substantial increases in peritoneal eosinophils and neutrophils.[68] Combination of both factors retained the distinctive responses of the individual factors with significant enhancement of cell numbers in both locations.

This model system suggests that the organ distribution of cells stimulated to develop by different regulators differs and that combinations of regulators should achieve a wider tissue distribution of responding cells. It is also likely, in certain situations such as a local infection, that high levels of one CSF, such as GM-CSF, may be produced locally while systemic levels of G-CSF become elevated. In combination, this would represent a quite sophisticated system for focusing the additional cells being produced to the location where they are most required.

REGULATOR COMBINATIONS AND DIFFERENTIATION COMMITMENT

In addition to stimulation of cell production, regulators can also induce differentiation commitment in responding cells.

Some of the clearest examples of the advantages of regulator combinations concern the induction of differentiation commitment and/or maturation in leukemic cell lines. Thus, some level of differentiation commitment can be achieved in HL60 or U937 leukemic cells by GM-CSF, G-CSF, IL-6, or LIF when acting alone. However, combinations of two of more of these elicit enhanced suppression of clonogenic cells and this principle is observable with other leukemic cell lines.[69,70] Unlike the situation with mitotic signalling, it is not yet apparent whether different differentiating-inducing factors necessarily initiate a common signalling pathway. Indeed, in a recent study of the action of LIF, IL-6, and OSM on M1 myeloid leukemic cells, overexpression of the helix-loop-helix gene SCL resulted in clear differences in the subsequent response of these cells to signalling by these three regulators even though the receptors concerned share a common gp130 subunit.[71]

Again, there are situations when regulator combinations can be antagonistic. Thus, GM-CSF and M-CSF can compete in vitro for commitment of bipotential progenitor cells to the granulocyte or macrophage lineage.[37,72] However, it is likely that, in vivo, the reserve of stem and progenitor cells is sufficient to compensate for such competitive actions.

COMMENTS

For the various reasons discussed above, it seems incorrect to regard the multiplicity of hematopoietic regulators as representing a highly redundant control system of dubious value. There is increasing evidence of the necessity to use regulator combinations to achieve certain types of cell production and, for others, of the higher efficiency of multiple regulatory factors, even when their actions appear to exhibit considerable overlap. These advantages also include the ability to achieve more subtle localization of cells produced and to achieve the complex mixtures of the cells required in certain situations (Fig 3).

If the existence of multiple regulatory factors indicates subtlety rather than redundancy, more effort needs to be expended to determine exactly what is achievable by the use of regulator combinations. This requires in vitro experiments that are demanding to design and difficult to publish in digestible form. However, the situation for our clinical colleagues is much worse. Some 20 regulators are available in recombinant form for clinical testing and the resources of suitable research units and patients will be strained to accomplish the required trials on individual agents. If the reality is that regulators are more efficient and effective when used in combination, the situation becomes a logistical nightmare. Even using a single dosage schedule, there are

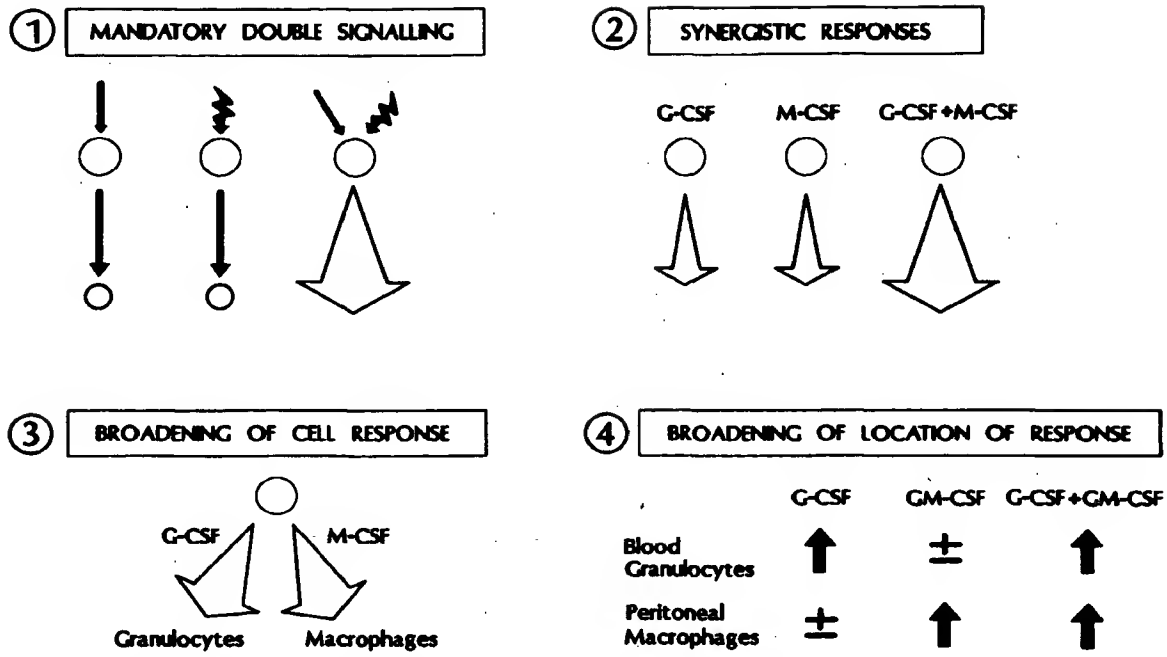


Fig 3. Four examples of the advantages of using multiple regulators. (1) Stem cells and some progenitor cells require double signalling for proliferation. (2) Combinations of factors can elicit superadditive synergistic proliferative responses. (3) Combination of two factors broadens the lineage range of maturing cells produced. (4) Combinations of factors can broaden the location of cells responding or accumulating after increased cell production.

more than 1 million possible combinations of 20 agents and testing of random combinations is impossible.

It may be possible to predict from in vitro data whether certain combinations might be of clinical value. Unfortunately, existing laboratory data are quite inadequate to cover all possibly valuable combinations and there is an urgent need to extend these laboratory data lest some quite unexpected favorable combinations are overlooked. A better understanding of the basic cellular principles involved in responses to regulators may help us in this dilemma.

REFERENCES

1. Metcalf D: The Hemopoietic Colony Stimulating Factors. Amsterdam, The Netherlands, Elsevier, 1984

2. Metcalf D, Burgess AW, Johnson GR, Nicola NA, Nice EC, DeLamarter J, Thatcher DR, Mermod J-J: In vitro actions on hemopoietic cells of recombinant murine GM-CSF purified after production in *Escherichia coli:* Comparison with purified native GM-CSF. J Cell Physiol 128:421, 1986

3. Metcalf D, Nicola NA: Proliferative effects of purified granulocyte colony-stimulating factor (G-CSF) on normal mouse hematopoietic cells. J Cell Physiol 116:198, 1983

4. Metcalf D, Nicola NA: The clonal proliferation of normal mouse hemopoietic cells: Enhancement and suppression by CSF combinations. Blood 79:2861, 1992

5. Metcalf D, Begley CG, Nicola NA, Johnson GR: Quantitative responsiveness of murine hemopoietic populations in vitro and in vivo to recombinant Multi-CSF (IL-3). Exp Hematol 15:288, 1987

6. Spivak JC: The mechanism of action of erythropoietin. Int J Cell Cloning 4:139, 1986

7. Ishibashi T, Koziol JA, Burstein SA: Human recombinant erythropoietin promotes differentiation of murine megakaryocytes in vitro. J Clin Invest 79:286, 1987

8. Kinashi T, Inaba K, Tsubata T, Tashiro K, Palacios R, Honjo T: Differentiation of an interleukin 3-dependent precursor B-cell clone into immunoglobulin-producing cells in vitro. Proc Natl Acad Sci USA 85:4473, 1988

9. Metcalf D: The leukemia inhibitory factor (LIF). Int J Cell Cloning 9:95, 1991

10. Kishimoto T: The biology of interleukin-6. Blood 74:1, 1989

11. Rose TM, Bruce AG: Oncostatin M is a member of a cytokine family that includes leukemia-inhibitory factor, granulocyte colony-stimulating factor and interleukin-6. Proc Natl Acad Sci USA 88:8641, 1991

12. Yang YC, Yin T: Interleukin-11 and its receptor. Biofactors 4:15, 1992

13. Metcalf D: Leukemia inhibitory factor. A puzzling polyfunctional regulator. Growth Factors 7:169, 1992

14. Metcalf D, Nicola NA, Gearing DP: Effects of injected leukemia inhibitory factor on hematopoietic and other tissues in mice. Blood 76:50, 1990

15. Ishibashi T, Kimura H, Shikama Y, Uchida T, Kariyone S, Hirano T, Kishimoto T, Takasuki F, Akiyama Y: Interleukin-6 is a potent thrombopoietic factor in vivo in mice. Blood 74:1241, 1989

16. Neben TY, Loebelenz J, Hayes L, McCarthy K, Stoudemire J, Schaub R, Goldman SJ: Recombinant human interleukin-11 stimulates megakaryocytopoiesis and increases peripheral platelets in normal and splenectomized mice. Blood 81:901, 1993

17. Sherr CJ: Colony-stimulating factor-1 receptor. Blood 75:1, 1990

18. Yarden Y, Kuang W-J, Yang-Feng T, Coussens L, Mune-

mitsu S, Dull TJ, Chen E, Schlessinger J, Francke U, Ullrich A: Human proto-oncogene c-kit: A new cell surface receptor tyrosine kinase for an unidentified ligand. EMBO J 6:3341, 1987

19. Bazan JF: Structural design and molecular evolution of a cytokine receptor superfamily. Proc Natl Acad Sci USA 87:6934, 1990

20. D'Andrea AD, Lodish HF, Wong GG: Expression cloning of the murine erythropoietin receptor. Cell 57:277, 1989

21. Gearing DP, King JA, Gough NM, Nicola NA: Expression cloning of a receptor for human granulocyte-macrophage colony-stimulating factor. EMBO J 8:3667, 1989

22. Kitamura T, Sato N, Arai K, Miyajima A: Expression cloning of the human IL-3 receptor cDNA reveals a shared beta subunit for the human IL-3 and GM-CSF receptors. Cell 66:1165, 1991

23. Fukunaga R, Ishizaka-Ikeda E, Seto Y, Nagata S: Expression cloning of a receptor for murine granulocyte colony-stimulating factor. Cell 61:341, 1990

24. Harada N, Castle BE, Gorman DM, Itoh N, Schreurs J, Barrett RL, Howard M, Miyajima A: Expression cloning of a cDNA encoding the murine interleukin 4 receptor based on ligand binding. Proc Natl Acad Sci USA 87:857, 1990

25. Takaki S, Tominaga A, Hitoshi Y, Mita S, Sonoda E, Yamaguchi N, Takatsu K: Molecular cloning and expression of the murine interleukin-5 receptor. EMBO J 9:4367, 1990

26. Yamasaki K, Taga T, Hirata Y, Yawata H, Kawanishi Y, Seed B, Taniguchi T, Hirano T, Kishimoto T: Cloning and expression of the human interleukin-6 (BSF-2/IFN beta 2) receptor. Science 241:825, 1988

27. Goodwin RG, Friend D, Ziegler SF, Jerzy R, Falk BA, Gimpel S, Cosman D, Dower SK, March CJ, Namen AE, Park LS: Cloning of the human and murine interleukin-7 receptors: Demonstration of a soluble form and homology to a new receptor superfamily. Cell 60:941, 1990

28. Gearing DP, Thut CJ, VandenBos T, Gimpel SD, Delaney PB, King JA, Price V, Cosman D, Beckmann MP: Leukemia inhibitory factor receptor is structurally related to the IL-6 signal transducer, gp130. EMBO J 10:2839, 1991

29. Cosman D, McKereghan KN, Alpert AR, Grabstein K, Ceretti DP: Cloning and expression of human and mouse IL-2 receptor cDNAs. Lymphokines 13:109, 1987

30. Miyajima A: Molecular structure of the IL-3, GM-CSF and IL-5 receptors. Int J Cell Cloning 10:126, 1992

31. Taga T, Hibi M, Hirata T, Yamasaki K, Yasukawa K, Matsuda T, Hirano T, Kishimoto T: Interleukin 6 triggers the association of its receptor with a possible signal transducer gp130. Cell 58:573, 1989

32. Gearing DP, Comeau MR, Friend DJ, Gimpel SD, Thut CJ, McGourty J, Brasker KK, King JA, Gillis S, Mosley B, Ziegler SF, Cosman D: The IL-6 signal transducer gp130: An oncostatin M receptor and affinity converter for the LIF receptor. Science 255:1434, 1992

33. Taga T, Narazaki M, Yasukawa K, Saito T, Miki D, Hamaguchi M, Davis S, Shoyab M, Yancopoulos CD, Kishimoto T: Functional inhibition of hematopoietic and neurotrophic cytokines by blocking the interleukin 6 signal transducer gp130. Proc Natl Acad Sci USA 89:10998, 1992

34. Byrne PV, Guilbert LJ, Stanley ER: Distribution of cells bearing receptors for a colony-stimulating factor (CSF-1) in murine tissues. J Cell Biol 91:848, 1981

35. Nicola NA, Metcalf D: Binding of ^{125}I-labeled granulocyte colony-stimulating factor to normal murine hemopoietic cells. J Cell Physiol 124:313, 1985

36. Nicola NA, Metcalf D: Binding of iodinated multipotential colony-stimulating factor (interleukin 3) to murine bone marrow cells. J Cell Physiol 128:180, 1986

37. Metcalf D, Burgess AW: Clonal analysis of progenitor cell commitment to granulocyte or macrophage production. J Cell Physiol 111:275, 1982

38. Walker F, Nicola NA, Metcalf D, Burgess AW: Hierarchical down-modulation of hemopoietic growth factor receptors. Cell 43:269, 1985

39. Jacobsen SE, Ruscetti FW, Dubois CM, Wine J, Keller JR: Induction of colony-stimulating factor receptor expression on hematopoietic progenitor cells: Proposed mechanism for growth factor synergism. Blood 80:678, 1992

40. Zsebo KM, Wypych J, McNiece IK, Lu HS, Smith KA, Kakare SB, Sachdev RK, Yuschenkoff VN, Birkett NC, Williams LR, Satyagal VN, Tung W, Bosselman RA, Mendiaz FA, Langley KE: Identification, purification and biological characterization of hematopoietic stem cell factor from Buffalo rat liver-conditioned medium. Cell 63:195, 1990

41. Yamaguchi Y, Suda T, Suda J, Eguchi M, Miura Y, Harada N, Tominaga A, Takatsu K: Purified interleukin 5 supports the terminal differentiation and proliferation of murine eosinophilic precursors. J Exp Med 167:43, 1988

42. Ishibashi T, Kimura H, Uchida T, Kariyone S, Friese P, Burstein SA: Human interleukin 6 is a direct promoter of maturation of megakaryocytes in vitro. Proc Natl Acad Sci USA 86:5953, 1989

43. Metcalf D: The colony-stimulating factors: Discovery to clinical use. Phil Trans R Soc Lond B 333:147, 1991

44. Schooley JC, Garcia J-F: Immunologic studies on the mechanism of action of erythropoietin. Proc Soc Exp Biol Med 110:636, 1962

45. Hammond WP, Csiba E, Canin A, Hockman H, Souza LM, Layton JE, Dale DC: Chronic neutropenia: A new canine model induced by human G-CSF. J Clin Invest 87:704, 1991

46. Yoshida H, Hayashi S-I, Kunisada T, Ogawa M, Nishikawa S, Okamura H, Sudo T, Schultz LD, Nishikawa S-I: The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. Nature 345:442, 1990

47. Wiktor-Jedrzejczak W, Urbanowska E, Aukerman S, Pollard JW, Stanley ER, Ralph P, Ansari AA, Sell KW, Szperl M: Correction by CSF-1 of defects in the osteopetrotic op/op mouse suggests local, developmental and humoral requirements for this growth factor. Exp Hematol 19:1049, 1991

48. Stewart CL, Kaspar P, Brunet LJ, Bhatt H, Gadi I, Köntgen F, Abbondanzo SJ: Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. Nature 359:76, 1992

49. Escary JL, Perreau J, Dumenil D, Ezine S, Brulet P: Leukaemia inhibitory factor is necessary for maintenance of haematopoietic stem cells and thymocyte stimulation. Nature 63:361, 1993

50. Croy BA, Guilbert LJ, Brown MA, Gough NM, Stinchcomb DT, Reed N, Wegmann TG: Characterization of cytokine production by the metrial gland and granulated metrial gland cells. J Reprod Immunol 19:149, 1991

51. Begg SK, Radley JM, Pollard JW, Chrisholm OT, Stanley ER, Bertoncello I: Delayed hematopoietic development in osteopetrotic (op/op) mice. J Exp Med 177:237, 1993

52. Li CL, Johnson GR: Rhodamine 123 reveals heterogeneity within murine Lin$^-$Sca-1$^+$ hemopoietic stem cells. J Exp Med 175:1443, 1992

53. Miura N, Okada S, Zsebo KM, Miura Y, Suda T: Rat stem cell factor and IL-6 preferentially support the proliferation of c-kit–positive murine hemopoietic cells rather than their differentiation. Exp Hematol 21:143, 1993

54. Migliaccio G, Migliaccio AR, Valinsky J, Langley K, Zsebo K, Visser JWM, Adamson JW: Stem cell factor induces proliferation and differentiation of highly enriched murine hematopoietic cells. Proc Natl Acad Sci USA 88:7420, 1991

55. Bot FJ, Van Eijk L, Schipper P, Backx B, Löwenberg B:

Synergistic effects between GM-CSF and G-CSF on highly enriched human marrow progenitor cells. Leukemia 4:325, 1990

56. McNiece IK, Stewart FM, Deacon DM, Quesenberry PJ: Synergistic interactions between hematopoietic growth factors as detected by in vitro mouse bone marrow colony formation. Exp Hematol 16:383, 1991

57. Metcalf D: The molecular control of cell division, differentiation commitment and maturation in haemopoietic cells. Nature 339:27, 1989

58. McNiece IK, Langley K, Zsebo KM: Recombinant human stem cell factor (rhSCF) synergises with GM-CSF, G-CSF, IL3 and Epo to stimulate human progenitor cells of myeloid and erythroid lineages. Exp Hematol 19:226, 1991

59. Metcalf D, Nicola NA: Direct proliferative actions of stem cell factor on murine bone marrow cells in vitro. Effects of combination with colony-stimulating factors. Proc Natl Acad Sci USA 88:6239, 1991

60. Metcalf D: Lineage commitment of hemopoietic progenitor cells in developing blast cell colonies: Influence of colony stimulating factors. Proc Natl Acad Sci USA 88:11310, 1991

61. Metcalf D: The cellular basis for enhancement interactions between stem cell factor and the colony stimulating factors. Stem Cells 11:1, 1993 (suppl 2)

62. Dührsen U, Villeval J-L, Boyd J, Kannourakis G, Morstyn G, Metcalf D: Effects of recombinant human granulocyte-colony stimulating factor on hemopoietic progenitor cells in cancer patients. Blood 72:2074, 1988

63. Cynshi O, Satoh K, Shimonaka Y, Hattori K, Nomura H, Imai N, Hirashima K: Reduced response to granulocyte colony-stimulating factor in W/W^v and Sl/Sl^d mice. Leukemia 5:75, 1991

64. Gliniak BC, Rohrschneider LR: Expression of M-CSF receptor is controlled post-transcriptionally by the dominant actions of GM-CSF or Multi-CSF. Cell 63:1073, 1990

65. Kelso A, Gough NM: Coexpression of granulocyte-macrophage colony-stimulating factor, γ-interferon and interleukins-3 and 4 is random in murine alloreactive T lymphocyte clones. Proc Natl Acad Sci USA 85:9189, 1988

66. Seelentag WK, Mermod J-J, Montesano R, Vassalli P: Additive effects of interleukin 1 and tumor necrosis factor-α on the accumulation of the three granulocyte and macrophage colony-stimulating factor mRNAs in human endothelial cells. EMBO J 6:2261, 1987

67. Rennick D, Young G, Gemmell L, Lee F: Control of hemopoiesis by a bone marrow stromal cell clone: Lipopolysaccharide and interleukin-1–inducible production of colony-stimulating factors GM-CSF and G-CSF. Blood 69:682, 1987

68. Metcalf D, Begley CG, Williamson D, Nice EC, DeLamarter J, Mermod J-J, Thatcher D, Schmidt A: Hemopoietic responses in mice injected with purified recombinant murine GM-CSF. Exp Hematol 15:1, 1987

69. Maekawa T, Metcalf D: Clonal suppression of HL60 and U937 cells by recombinant leukemia inhibitory factor in combination with GM-CSF or G-CSF. Leukemia 3:270, 1989

70. Maekawa T, Metcalf D, Gearing DP: Enhanced suppression of human myeloid leukemic cell lines by combinations of IL-6, LIF, GM-CSF and G-CSF. Int J Cancer 45:353, 1989

71. Tanigawa T, Elwood N, Metcalf D, Cary D, DeLuca E, Nicola NA, Begley CG: The SCL gene product is regulated by and differentially regulates cytokine responses during myeloid leukemic cell differentiation. Proc Natl Acad Sci USA 90:7864, 1993

72. Metcalf D, Merchav S, Wagemaker G: Commitment by GM-CSF or M-CSF of bipotential GM progenitor cells to granulocyte or macrophage formation, in Baum S, Ledney GD, Thierfelder S (eds): Experimental Hematology Today 1982. Basel, Switzerland, Karger, 1982, p 3